Mammary Gland Neoplasia in Long-Term Rodent Studies

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Breast cancer, the most frequent spontaneous malignancy diagnosed in women in the western world, is continuously increasing in incidence in industrialized nations. Although breast cancer develops in women as the result of a combination of external and endogenous factors such as exposure to ionizing radiation, diet, socioeconomic status, and endocrinologic, familial, or genetic factors, no specific etiologic agent(s) or the mechanisms responsible of the initiation of the disease has been identified as yet. Thus, experimental models that exhibit the same complex interactions are needed for testing various mechanisms and for assessing the carcinogenic potential of given chemicals. Rodent mammary carcinomas represent such a model to a great extent because, in these species, mammary cancer is a multistep complex process that can be induced by either chemicals, radiation, viruses, or genetic factors. Long-term studies in rodent models have been particularly useful for dissecting the initiation, promotion, and progression steps of carcinogenesis. The susceptibility of the rodent mammary gland to develop neoplasms has made this organ a unique target for testing the carcinogenic potential of specific genotoxic chemicals and environmental agents. Mammary tumors induced by indirect- or direct-acting carcinogens such as 7,12-dimethylbenz(*a*)anthracene or *N*-methyl-*N*-nitrosourea are, in general, hormone-dependent adenocarcinomas whose incidence, number of tumors per animal, tumor latency, and tumor type are influenced by the age, reproductive history, and endocrinologic milieu of the host at the time of carcinogen exposure. Rodent models are informative in the absence of human data. They have provided valuable information on the dose and route of administration to be used and optimal host conditions for eliciting maximal tumorigenic response. Studies of the influence of normal gland development on the pathogenesis of chemically induced mammary carcinomas have clarified the role of differentiation in cancer initiation. Comparative studies with the development of the human breast and the pathogenesis of breast cancer have contributed to validate rodent-to-human extrapolations. However, it has not been definitively established what type of information is necessary for human risk assessment, whether current toxicity testing methodologies are sufficient for fulfilling those needs, or whether treatment-induced tumorigenic responses in rodents are predictive of potential human risk. An alternative to the traditional bioassays are mechanism-based toxicology and molecular and cellular approaches, combined with comparative *in vitro* systems. These approaches might allow the rapid screen of chemicals for setting priorities for further studies to determine the dose–response relationship for chemical effects at low doses, to assess effects other than mutagenesis and/or tumorigenesis, or to establish qualitative and quantitative relationships of biomarkers to toxic effects. Until there is enough information on the predictive value of mechanism-based toxicology for risk assessment, this approach should be used in conjunction with and validated by the traditional *in vivo* long-term bioassays. *Key words*: breast cancer, chemically induced mammary carcinogenesis, endocrine system, hormones, rodent mammary gland, rodent tumor model. *Environ Health Perspect* 104:938–967 (1996)

The mammary gland, a specialized accessory gland of the skin that characterizes the mammalian species, is a frequent source of tumors or neoplasms. The terms tumor and neoplasm are applied indistinctly to either benign or malignant lesions because tumor (from the Latin *tumere*, to swell) means any pathological enlargement or new growth, as does neoplasm (from the Greek *neos* new + *plasma* formation) (*1*); however, neither term defines the true nature of a given growth. Benign tumors are those that do not invade adjacent tissues, do not metastasize to distant sites, and can be cured by local excision. Malignant tumors, or cancer, are neoplasms characterized by their ability to invade, metastasize, and ultimately cause the death of the host. They are called carcinomas when they are derived from epithelial cells, or sarcomas if they are mesenchymal in origin (*2*). The emphasis given here to the classification of tumors as benign or malignant is due to the implications of the diagnosis of malignancy because it affects the epidemiology of the disease, the clinical and therapeutic approaches, and the interpretation of experimental data.

The most significant malignant neoplasm in the human breast is the adenocarcinoma. The understanding of this disease requires a multiprong approach that encompasses epidemiologic and clinical data, as well as adequate experimental systems for evaluating risk assessment or for testing chemopreventive and therapeutic agents.

Breast cancer is the most frequent spontaneous malignancy diagnosed in women in the western world. In the United States alone, 182,000 new cases were diagnosed in 1994; its incidence has been increasing for several decades (*3–5*). Data from continuously operating cancer registries, such as the one in the state of Connecticut and the Surveillance, Epidemiology, and End Results (SEER) program established by the National Cancer Institute in 1973 (which collects data from nine population-based cancer registries covering about 10% of the US population), reveal that the age-adjusted incidence of breast cancer rose at a rate of 1.2%/year from 1940 to 1980; it rose even more steeply in the next 7 years, reaching a peak of 112.4 cases/100,000 women in 1987, although it decreased to 104.6 cases/100,000 women by 1989 (*3–5*). The increased incidence of cancer has been partly attributed to an improvement in early detection, as indicated by the progressively increased percentage of patients with early stage (0 to I) disease (from 45.2% in 1985–1986 to 53.1% in 1991) and decreased percentage of patients in stage II and III disease (*6*). Early diagnosis has improved the rates of cure and prolonged survival; however, stage-specific survival rates have increased only slightly since the mid-1970s, and breast cancer remains only second to lung cancer as a cause of death, causing 46,000 deaths in 1994 (*3–5*).

From all experimental systems available for the study of mammary cancer, rodent models have been particularly useful, mainly because spontaneous mammary tumors are frequently observed in long-term studies (*7*). They are the most common hormone-dependent spontaneous neoplasms developed in several strains of female rats; their development, which increases with aging, is attributed to hormonal imbalances such as the constant estrous that occurs in old Sprague-Dawley rats (*7–9;* JT Stevens, personal communication). In mice, spontaneous mammary tumors are either linked to the infection of females with an exogenous

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mouse mammary tumor virus (MMTV) or a less virulent endogenous provirus (*9–11*). Nevertheless, mammary tumors are also hormone dependent, as initially demonstrated by Lathrop and Loeb, who reported that ovariectomy at an early age either inhibited or delayed the appearance of mammary tumors in outbred mice (*9*).

The susceptibility of the rodent mammary gland to develop neoplasms has made this organ a unique target for testing the carcinogenic potential of specific chemicals. Several carcinogens that induce mammary tumors in both mice and rats have been extensively studied in both species (*9,12–36*). Tumors induced by administration of chemical carcinogens constitute useful tools for dissecting the multistep process of carcinogenesis, which involves initiation, promotion, and progression, and serve as a baseline for testing the carcinogenic potential of chemicals in risk assessment (*7,37*). Chemically induced mammary tumors are, in general, hormone-dependent adenocarcinomas. Their incidence, number of tumors per animal, and tumor type are influenced by the age of the host at the time of carcinogen exposure, reproductive history, endocrinologic milieu, and diet, among other factors. These factors, in turn, influence the development and degree of mammary gland differentiation (*38–41*), which are subject to a multiplicity of endocrine stimulatory and inhibitory influences from embryogenesis onward (*39,40,42–44*). If these influences are not exerted in proper temporal, sequential, and quantitative relationships, normal development, differentiation, and function are adversely affected (*41*). The development of the mammary gland, in turn, cannot be separated from its aging, a process that markedly influences the incidence of spontaneous tumors in all strains of rats (*8,45,46*), as well as in mice infected with MMTV (*10*).

The ideal animal tumor model should mimic the human disease (*23,47*) by providing a model for ascertaining the influence of host factors on the initiation of tumorigenesis, mimicking the tumorigenic response elicited under specific age and reproductive conditions, and determining the response of the tumors induced to chemotherapy. The use of experimental models of mammary carcinogenesis in risk assessment requires that the influence of ovarian, pituitary, and placental hormones, as well as overall reproductive events, and age at menarche and menopause are taken into consideration because they are important modifiers of the susceptibility of the organ to neoplastic development (*9,41,48*). Several species such as rodents (*9,41*), dogs (*49*), cats (*50*), and monkeys (*51*) have been evaluated for these purposes; however, none of them fulfill all the criteria specified above. Rodents, however, are the most widely used models (*24–36,41,47, 52*). Therefore, this work will discuss various aspects of the rodent model such as 1) the normal anatomy and development of the mammary gland during reproductive events and aging; 2) the influence of reproductive, aging, and nongenotoxic factors on tumor development; 3) experimental genotoxic-induced rodent mammary tumor models; 4) modifiers of susceptibility of the gland to neoplasia; and 5) impact of hormones and growth factors on mammary carcinogenesis. In addition, current knowledge on human breast cancer will be briefly reviewed to establish a unifying concept between human and rodent carcinogenesis.

Mammary Gland Development

Anatomy of the Mammary Gland

Gross anatomy. The mammary gland, whose function is the secretion of milk for the nourishment of the offspring, adapts to this function primarily by developing a number of glands adequate for sustaining the number of newborns that are characteristic for a given species (*41,47,53*). In both rats and mice, the mammary glands are aligned ventrolaterally along the mammary or milk lines from the cervical to the inguinal regions. The only externally visible portion of the mammary gland in the female is the nipple, a cutaneous structure located ventrolaterally, and from which the rest of the mammary gland extends dorsolaterally as flat subcutaneous sheets of fibroadipose tissue (*40,53*). Male mice have no nipples, and they usually have four pairs of rudimentary glands consisting of major branching ducts with no alveoli, primary ducts, or openings to the exterior (*53*). Female mice, on the other hand, have five pairs of mammary glands, one cervical, two thoracic, and two abdominal–inguinal pairs. The female rat has six pairs, the fifth and sixth pairs being located in the inguinal region. Except for the difference in total number of mammary glands in the females, the anatomic location and distribution of these paired organs is similar between these two species; therefore, the descriptions below apply indistinctly to both, unless otherwise specified. No further description will be done of the mammary gland in males because it is an atrophic organ that rarely gives origin to spontaneous tumors or is the subject of carcinogenicity studies.

The size and distribution of the mammary glands vary markedly as a function of sex, age, hormonal stimulation, and reproductive condition (*23,39,40,54*). Classical descriptions of mammary gland distribution apply to the glands of fully mature parous females in which the first pair, or cervical mammary glands, extend cranially to the parotid and mandibular salivary glands and laterally along the medial aspect of the forelimb, reaching the interscapular fat pad. The second and third pairs are located in the thoracic region, extending laterally and dorsally from the nipples, which are medially located. The two pairs of abdominal–inguinal glands in the female mouse and the three pairs of abdomino–inguinal glands in the female rat are in

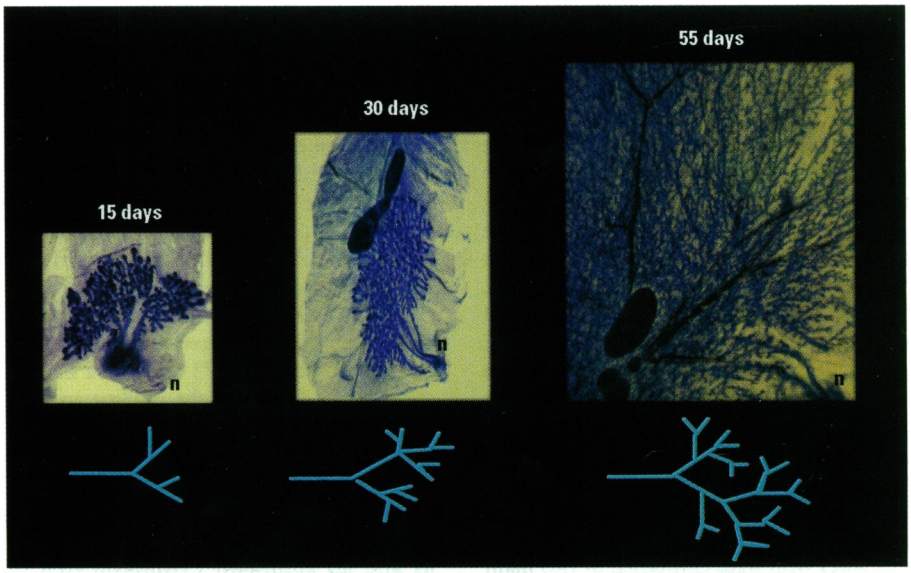

Figure 1. Development of the fourth (abdominal) mammary gland of the female Sprague-Dawley rat. Comparison of gland area and number and complexity of branching at 15 (×3.9), 30 (×3.4), and 55 (×5.6) days of age. n, nipple. Whole mount preparations, toluidine blue (×3).

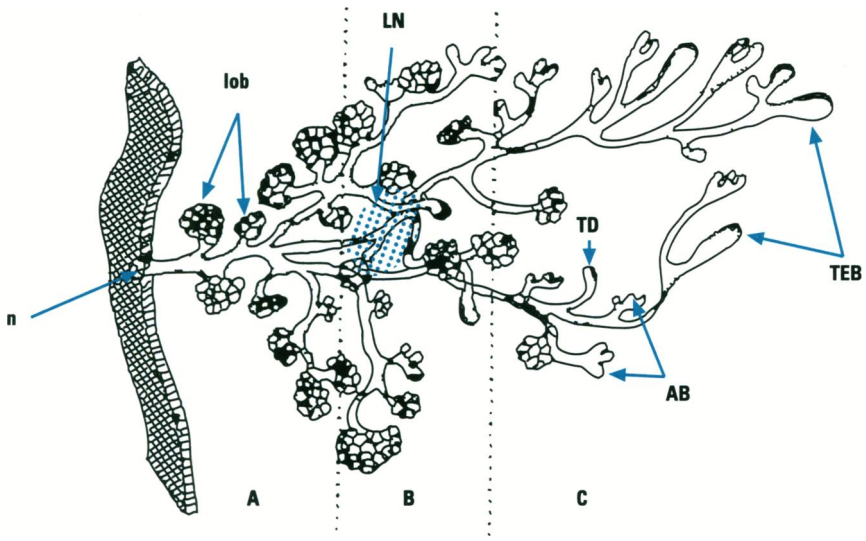

Figure 2. Schematic representation of the fourth (abdominal) mammary gland of a 55 day-old virgin female rat. The gland was divided into three zones: A, proximal to the nipple (n), contains more numerous lobules type 1 (lob). Zone B, medial, encompasses the lymph node (LN). Zone C, distal to the nipple, contains the majority of actively growing terminal end buds (TEB); alveolar buds (AB) and terminal ducts (TD) are also present. From Russo and Russo (*20*).

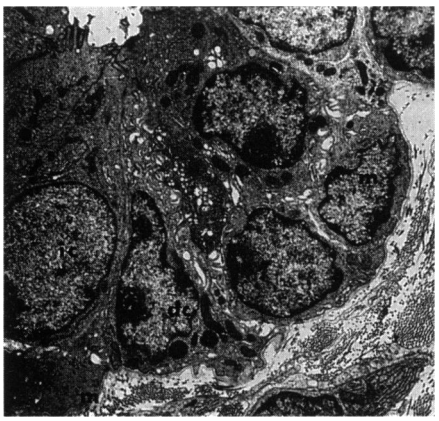

Figure 3. Ultrastructure of a rat mammary gland ductule. The epithelium is composed of intermediate (ic) and dark cells (dc) resting on a discontinuous layer of myoepithelial cells (m). Uranyl acetate-lead citrate (×1400). From Russo et al. (*40*).

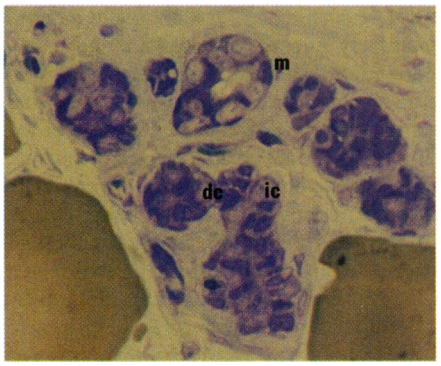

Figure 4. Lobule type 1 of the virgin rat mammary gland. Each ductule or alveolus is lined by a single layer of low cuboidal epithelium composed of myoepithelial (m), intermediate (ic), and dark cells (dc). Plastic embedded 1 µm section toluidine blue (×270).

close continuity, extending medially in the pubic region, caudally to the perianal region, and laterally onto the medial aspect of the hindlimbs (*40,47,53–55*).

Microscopic anatomy. The basic architecture of the mammary gland has been extensively described as a complex structure composed of parenchyma and stroma (*40,42,44*). The understanding of the structure of the gland requires the study of the complete organ in whole mount preparations (Fig. 1) combined with histological sections representative of specific areas of the same because different topographic areas contain structures that differ in their morphology, cell kinetic characteristics, hormone responsiveness, and carcinogenic potential (*23*).

The parenchyma consists of one or two major lactiferous ducts that grow from the nipple into the surrounding fat pad. The growth of the mammary gland occurs as the result of a combined process of lengthening of major ducts in a straight fashion in the areas closer to the nipple, which in the rat has been called zone A (Fig. 2) (*39,40, 54,55*). A progressive dichotomous and sympodial branching of smaller ducts and lateral buds occurs more frequently in the middle third of the gland (zone B). The portion of the gland opposite to the nipple (zone C) contains the most actively growing terminal ductal structures, the terminal end buds (TEBs) (Fig. 2) (*15,22,23,39,40, 42,53*). The mammary ducts are tubular structures with walls composed of two main cell types, an internal layer of epithelial cells lining the lumen and an external and discontinuous layer of myoepithelial cells resting on the basement membrane (Fig. 3,4) (*40*). Both the thickness of the epithelial layer and its degree of differentiation vary as a function of the specific structure in which they are located. Major ducts tend to be lined by a single or pseudostratified layer of low columnar or cuboidal epithelial cells. The luminal border is fringed with short, blunt microvilli. Adjacent cells interdigitate and are joined by well-developed junctional complexes (*42,56–58*). TEBs, on the other hand, are lined by a multilayered epithelium composed of large cuboidal cells exhibiting a high rate of cell proliferation (Fig. 5) (*40*). In the mouse, the end bud consists of four to six layers of cuboidal epithelium. On the basal surface of the end bud beneath the basal lamina are the cap cells (*59*). These cells lack differentiated features: they lack cytoplasmic polarity, they do not form cell junctions, and they do not contain highly organized cytoskeletal elements. These cells are interpreted to represent a pluripotent stem cell population, capable of differentiating into both mammary ductal and mammary myoepithelial cell types (*42*). Ultrastructurally, the cells composing the TEBs have been described as intermediate cells (*6,57*) due to the moderate electron density of the nucleus and the cytoplasm, in contrast with the other two cell types described in both the rat and the mouse mammary glands, the dark and the light cells (*42,57–60*). Intermediate cells (Fig. 3–6) have a large round or oval nucleus and an abundant cytoplasm rich in organelles, but show almost no secretory activity. In contrast, dark cells (Fig. 3–6), which are more numerous in ducts and secretory alveoli, have smaller and convoluted nuclei and contain abundant cytoplasm rich in ribosomes, Golgi complexes, lipid droplets, and secretory vacuoles (*40*). The external layer or myoepithelium is located between the epithelial layer and the basal lamina (Fig. 3,6). Myoepithelial cells appear moderately electron dense due to the presence of thick bundles of tonofilaments and myofibril-like bundles of actin and myosin in the cytoplasm, a characteristic that these cells share with smooth muscle fibers. Their plasma membrane contains numerous pinocytotic vesicles and is attached to the basal lamina by hemidesmosomes (Fig. 3,6) (*40*). The basement membrane on which the epi-myoepithelial complex rests is composed of a bilayered basal lamina: the lamina lucida, in close contact with the myoepithelium, and the lamina densa, which forms a continuous sheath between the lamina lucida and the stroma (*42,48,61–63*).

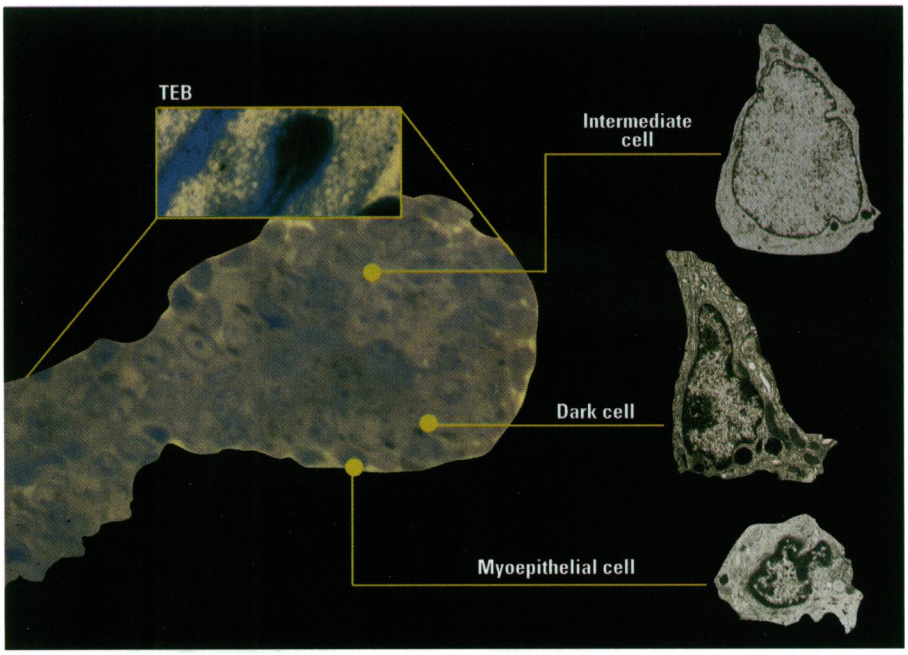

Figure 5. Composite showing the terminal end bud (TEB) of the virgin rat mammary gland in whole mount (×50), longitudinal section of a plastic embedded TEB stained with toluidine blue (center, ×1395); electron micrograph of intermediate (×3575), dark (×7125), and myoepithelial cells (×7500). Uranyl acetate-lead citrate.

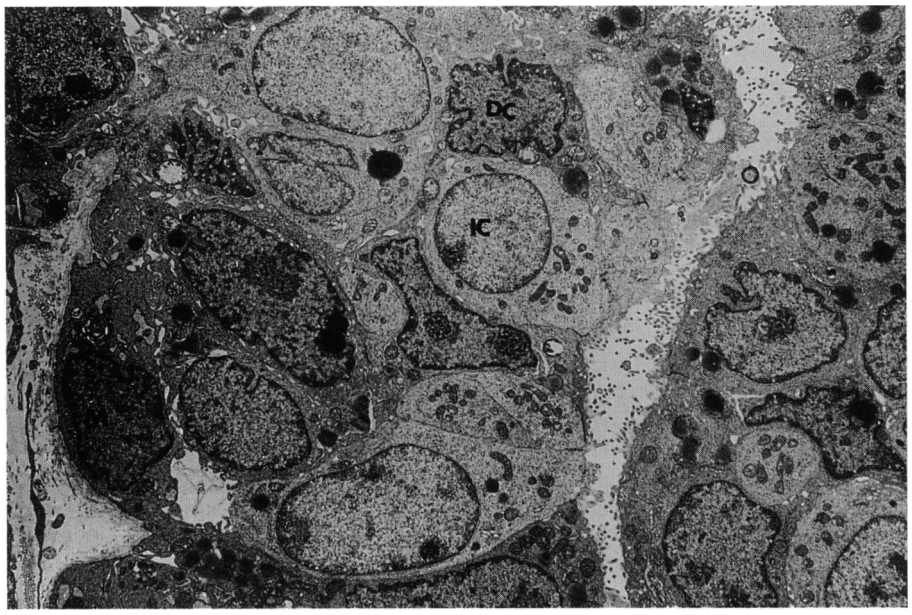

Figure 6. Electron micrograph of the terminal end bud of the virgin rat mammary gland. Myoepithelial cells (M) rest on the electron dense basal lamina. Dark cells (DC) and intermediate cells (IC) form the multilayered epithelium and emit microvilli toward the lumen. Uranyl acetate-lead citrate (×2275). From: Russo et al. (*40*).

Prenatal and Postnatal Development

The developmental and functional states of the mammary gland are subject to a multiplicity of genetically determined endocrine stimulatory and inhibitory influences from early embryogenesis through the senescence of the individual. Studies of the sex differences in mammary gland histogenesis indicate that the rodent mammary rudiment is sensitive to the influences of gonadal steroids during the prenatal stage (*44,64,65*). In mice, sexual differentiation of the gonads occurs on the late 13th day of gestation, after which the testes start to produce androgens in the male embryo. On the 14th day, the sexual phenotype of the mammary gland is determined (*44*). At this time, the male mammary bud becomes constricted at its epidermal junction and ultimately becomes completely detached. X-ray destruction of the fetal testes on the 13th day of gestation causes the mammary bud of the male to remain attached to the epidermis and the duct primordia to ramify similarly to the primordia of the female. Testosterone injections into pregnant animals cause the mammary buds of female embryos to undergo male-type development (*44*). The milk-fed newborn is exposed to a rich source of growth factors; these factors might play important roles in the growth and development of the mammary gland (*66*). One of the principal growth factors present in milk is epidermal growth factor (EGF), which is an important regulator of mouse mammary epithelial cell proliferation and differentiation both *in vivo* and *in vitro* (*67*).

In the Sprague-Dawley rat, the mammary gland evolves from a primary main lactiferous duct; it branches into multiple secondary ducts, whose length and the number of branches it originates increase with aging of the animal (Fig. 1). By the second week of postnatal life, the ducts have further sprouted to a sixth generation of branches, which end in club-shaped TEBs composed of three to six layers of medium-sized epithelial cells (Fig. 5,6). The number of TEBs is maximal when the rats are 21 days old; after this age, TEBs begin to cleave into three to five smaller buds or alveolar buds (ABs). The progressive differentiation of TEBs into ABs is accentuated by each estrous cycle, which starts when the animals are 30–42 days old (*39*). In the nonpregnant female, the development of the mammary gland is rigorously controlled by the ovary. Ovariectomy causes regression of end buds and cessation of growth (*42*). The ovary, in turn, depends on pituitary gonadotropins for its development and function. Pituitary luteinizing hormone (LH) and follicle stimulating hormone (FSH) interact with growth hormone (GH) and prolactin (PRL) to modulate ovarian steroidogenesis, as well as the secretion of nonsteroidal glycoproteins such as inhibin and activin (*68–71*). In general, estrogens are responsible for growth of mammary ducts and progesterone is necessary for lobuloalveolar growth in the mouse (*72*); however, a direct mitogenic effect of estrogens on the mammary gland has not been clearly demonstrated (*73*). A postulated mechanism is that estrogens stimulate secretion of growth factors such as EGF (*74*) or other growth factors of mammary or extramammary origin that might sensitize the mammary gland to mitogenic factors (*73,75*). Estrogens, on the other hand, have a mitogenic effect on several human breast cancer cell lines. When estrogens and progesterone are injected together, lobuloalveolar develop-

ment is promoted, with inhibition of lactogenesis, probably through an indirect effect by antagonizing glucocorticoid action that blocks casein gene expression (*76,77*). The mammogenic effects of ovarian steroids are largely dependent on the integrity of the pituitary gland, because the effects of estrogen and progesterone cannot be demonstrated in hypophysectomized animals (*78*). Ductal elongation and branching that occurs during puberty is positively regulated by ovarian estrogens and pituitary GH, which in turn might act through its local mediator, insulin-like growth factor I (IGF-I) (*66*). Furthermore, in the rat, estrogens stimulate the secretion of pituitary PRL; this hormone, rather than progesterone, appears to regulate the lobuloalveolar development of the mammary gland and exerts a direct effect on the growth of the mammary parenchyma and epithelium (*67,79*).

Mammary Gland Development during Pregnancy

Pregnancy represents a completely novel endocrinologic experience to the female organism. The fertilized egg becomes a new endocrine organ soon after conception. The developing blastocyst and the placenta represent a rich source of hormones and growth factors, which, in conjunction with fetal secretions, enter the maternal bloodstream, thus influencing multiple target organs (*39*). In the first pregnancy of a young female rat, there are six pairs of mammary glands that differ in their degree of development, depending upon the topographic location of each specific gland and of the distance between the location of a given parenchymal structure and the nipple (Fig. 2) (*39,80*). Gland development is also influenced by other factors, such as the number of ovulatory cycles that have occurred after the initiation of the ovarian function, diet and specific genetic characteristics of the animal (*39,81,82*).

At mating, the mammary glands contain a large proportion of TEBs, which are more numerous in the zone C of the glands (Fig. 2,6,7A,8A). They are also more numerous in thoracic than abdominal mammary glands. There are also ABs, but few lobules are present (*39*). Cervical stimulation at mating increases PRL levels, which in turn increase the number of ovarian luteal LH receptors, thus enhancing steroidogenesis. Early in pregnancy, the combined influences of ovarian estrogen, progesterone, and inhibin (*68,71*), with the production of rat chorionic gonadotropin (rCG) and rat placental lactogen (rPL) by the developing embryo, contribute to stimulate the mammary glands to undergo active cell proliferation.

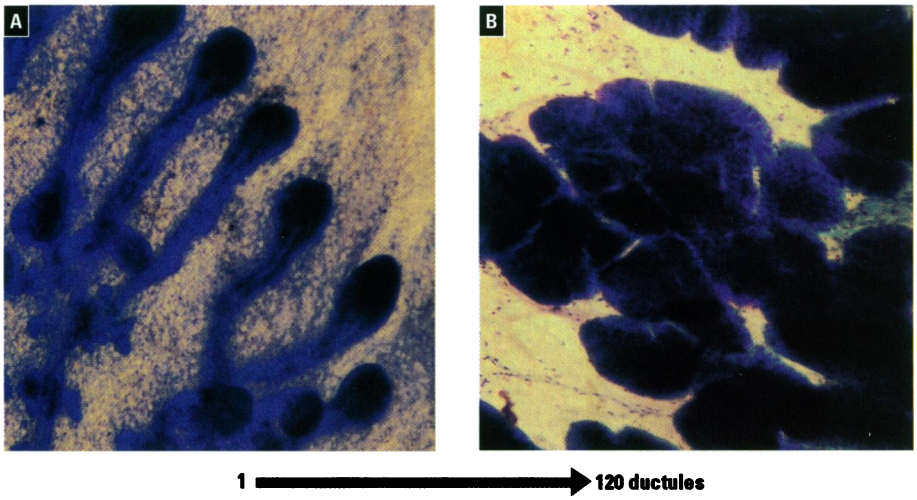

Figure 7. (A) Terminal end buds (TEBs) located in Zone C of the mammary gland of a 55 day-old virgin rat. (B) Lobules type 3 and 4 located in zone C of the mammary gland at the end of pregnancy. Whole mount preparations, toluidine blue (×150).

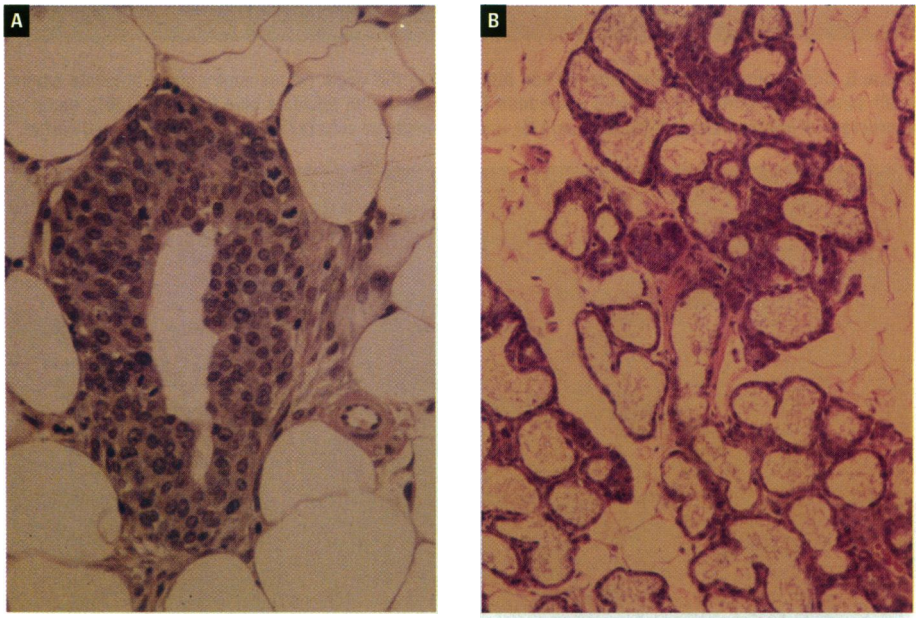

Figure 8. (A) Histological section of the terminal end buds shown in Figure 7A showing a multilayered epithelium and a narrow centrally located lumen (×362). (B) Lobules type 3 and 4 shown in Figure 7B are composed of secretory alveoli lined by a single layer of low cuboidal epithelial cells; the lumen of the alveoli is distended and milk filled (×395) H&E.

Greater activity is observed in TEBs, which rapidly cleave to form ABs, thus diminishing their number. ABs, in turn, progressively differentiate into lobules (*39*). In early pregnancy, the estrogen receptor content of the mammary gland becomes significantly higher than that of virgin rats (*83*). Pituitary relaxin, which plays an important role in the development of the nipples during the second half of pregnancy, also influences the development of lobules and the lactational capacity of the mammary glands (*84*).

During the gestational process, immunocytochemical reactions with antilaminin and type IV collagen antibodies reveal that the basement membrane remains intact around the newly formed lobules. Myosin-positive myoepithelial cells are attenuated and discontinuous in the alveoli, while they are prominent in the main ducts (*62,63*). The alveolar epithelium exhibits positive immunoreactivity with inhibin antibodies, which reaches its peak by the 15th day of pregnancy. Thereafter, the reaction becomes more intense in the perilobular stroma (*85,86*). At the end of pregnancy, the mammary gland is almost completely composed of densely packed lobules (Fig. 7B,8B). They

Table 1. Density of terminal end buds (TEB), terminal ducts (TD), alveolar buds (AB), and lobules in the rat mammary gland

Groups	Age (days)	TEB	TD	AB	Lobules
Young virgin	55	5.4 ± 0.3	5.5 ± 0.3	15.8 ± 2.1	1.2 ± 0.3
Old virgin	180	0.6 ± 0.2	6.0 ± 2.1	14.8 ± 2.0	0.8 ± 1.2
Multiparous	180	0.0	0.7 ± 0.8	14.2 ± 4.5	3.4 ± 1.6

Age in days at the time of 7,12-dimethylbenz(*a*)anthracene administration. All values listed represent the mean ± SD of the number of structures per square millimeter of gland and reflect the structural state of the gland at the moment of DMBA administration.

Table 2. DNA labeling index of terminal end buds (TEB), terminal ducts (TD), and alveolar buds (AB)

	TEB		TD		AB	
Groups	Labeled cells	Labeled TEB	Labeled cells	Labeled TD	Labeled cells	Labeled AB
Young virgin	34.4 ± 7.6	100	12.3 ± 5.8	70	7.9 ± 3.3	50
Old virgin	14.8 ± 4.7	100	4.9 ± 3.4	28	10.9 ± 1.7	5
Multiparous	0.0	0	0.3 ± 0.5	8	0.3 ± 0.05	0.9

Labeled cells, number of cells incorporating ^{3}H-thymidine/100 cells; labeled TEB, percentage of TEB containing labeled cells; labeled TD, percentage of TD containing labeled cells; labeled AB, percentage of AB containing labeled cells. All labeled cell values listed represent the mean of percentages ± SD.

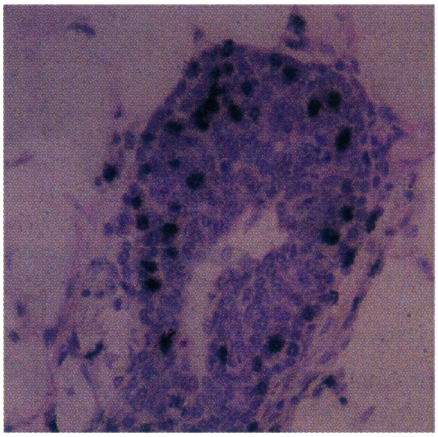

Figure 9. Autoradiogram of a terminal end bud located in Zone C of the mammary gland of a 55 day-old virgin rat shows active incorporation of ^{3}H-thymidine predominantly into ductal epithelial cells. NTB2 nuclear track emulsion, H&E (×333).

show active secretory activity in most acini. Lumina are distended by milk and the acinar lining epithelium has become a flattened single layer of low cuboidal cells (Fig. 8B), which stain positively with the antibody against milk fat globule membrane antigen (*62*).

Lactation and Post-lactational Involution

Immediately following parturition, the estrogen receptor content of the mammary gland, expressed as cytosolic estrogen receptor/g DNA increases significantly over a period of 2 to 4 days, which coincides with a period of DNA increase (*83*). Lactation, which lasts up to 3 weeks, delays the reinitiation of the estrous cycle and ovulation in postpartum rats (*68*). During lactation PRL levels are elevated, and the PRL receptor mRNAs increase significantly in the mammary glands, starting on the last day of pregnancy and continuing to increase during lactation (*87*). In the secretory acini of the lactating gland, myoepithelial cells are attenuated, with large gaps between cells (*63*). The basement membrane remains continuous, and fibronectin appears as a new component, with a distribution similar to that of type IV collagen and laminin. As in the mammary gland of pregnancy, epithelial cells stain positively with antibody against milk fat globule membrane antigen (*62*). After weaning, PRL levels return to normal and ovarian follicles develop into preovulatory size, secreting 17β-estradiol in quantities sufficient for inducing a preovulatory gonadotropin surge, which will resume the normal estrous cycle (*68*). The mammary gland exhibits an early increase in weight, which reaches a peak value at 24 hr after weaning. From then on, the weight declines, reaches the initial value at approximately the third day, and becomes very low by the 10th day post-weaning when most of the parenchyma is replaced by fat (*88*). The involution of the mammary gland is accompanied by profound morphological and physiological changes. The secretory alveolar structures collapse, with active removal of cells and secretions by macrophages. There is an increase in the lysosomal enzymes acid phosphatase and aryl sulfatase in both epithelial cells and macrophages (*88*). The collapsed alveolar structures form large irregular clusters that contain epithelial cells still reacting positively with milk fat globule membrane antigen. The myoepithelial cells become arranged in distorted circular structures, and the basement membrane acquires a broader appearance (*62*).

The Mammary Gland in the Aging Parous and Nulliparous Rat

Even though mammary gland involution after weaning is apparently completed after 10 days of pup removal, the architecture of the organ remains permanently modified. The mammary gland of an animal that has completed its first full term pregnancy and lactation retains a larger number of ABs and lobules. The comparative study of the architecture and cell kinetic characteristics of the mammary gland of a multiparous animal that has completed two pregnancies with lactation, with that of a virgin rat of the same age reveals that the involuted gland of the multiparous rat has a general appearance similar to that of the virgin animal. However, quantitative differences exist. By 40–42 days post weaning, the mammary glands of the multiparous rats do not contain TEBs. Terminal ducts (TDs) are occasionally seen; the number of ABs is about the same, and the number of lobules is four times higher than that observed in the age-matched virgin rats (Table 1).

Determination of the DNA labeling index (DNA-LI) by quantitation of the number of nuclei that had incorporated ^{3}H-thymidine (Fig. 9) revealed that 100% of the TEBs present in the mammary gland of the 180-day-old virgin rat contain proliferating cells that incorporate the radiolabeled precursor (*16–18*). However, the number of labeled cells per TEB is markedly lower than in young virgin rats (aged *40–50* days). Aging reduces the percentage of labeled cells from 34.4% (Fig. 8) to 14.8% (Table 2). The percentages of labeled TDs and their DNA-LI is also reduced in the old virgin rats. In the resting glands of multiparous rats, TDs are rarely observed and only 8.2% of them contain labeled cells, with a very low DNA-LI. ABs show notable differences in their DNA-LI in the three groups under study (Table 2). Young and old virgin rats have 50% and 5% of their ABs labeled, respectively. In the resting glands of multiparous rats, only 0.9% of the ABs are labeled and the number of cells incorporating DNA precursor is low. Lobular structures do not incorporate DNA precursor into their epithelial cells, and myoepithelial cells are only occasionally labeled (*16–18*). The profound morphological and cell kinetic differences observed between young and old virgin rats and between old virgin and multiparous rats are not the result of differences in circulating hormonal levels because the levels of PRL and estradiol are similar in the three groups of animals studied (*89*). However, with senescence, the circulating hormone levels

are markedly altered compared to young females (*90*); aged female Sprague-Dawley rats have significantly lower FSH, estradiol, and progesterone levels than young cycling females. PRL levels on the other hand have been shown to be elevated (*91*). These changes in hormone levels manifest themselves with a lengthening of cycle duration as a precursor to acyclicity that occurs in aged females (*92,93*). The binding of estrogen to receptors in the pituitary, hypothalamus, uterus, and mammary gland is decreased in aged noncycling rats (*94–97*).

Influence of Reproductive, Aging, and Nongenotoxic Factors on Tumor Development

Several hormones have a biologically significant influence on the normal development of the mammary glands, as well as significant effects on their normal functional states. These multiple hormonal influences act in concert for producing an integrated response under the control of the hypothalamus–anterior pituitary system, which plays primary roles in the development and function of the mammary gland (*98,99*). To evaluate the role of hormones in mammary gland neoplasia, it is helpful to review hormonal influences on the normal development and functional states of the gland. Much of what is known about the endocrine control and endocrine influences on the development of the mammary gland is derived from observations on the effect of hormone withdrawal through hypophysectomy, adrenalectomy, or gonadectomy or by manipulating tissue cultures (*64,65,100,101*). Despite the complexity of the endocrine systems, hormones can be classified into two major categories: those that are regulated by the central nervous system (CNS), namely, the hypothalamus–anterior pituitary system and those whose production is, for the most part, independent of the CNS, i.e., hormones of the peripheral system, which are regulated more directly by their own effects (*98*).

Hypothalamus–Anterior Pituitary System

The development, anatomy and physiology of the pituitary gland and its relationship with the hypothalamus have been extensively studied during the last two decades, especially in experimental animals, with the rat being the animal most frequently used (*98,102*). Although many pituitary hormones have a common evolutionary origin, extrapolations of findings obtained in different animal species to human conditions are difficult, especially due to differences in the development and function of the pituitary gland occurring with aging, a period in life of extreme importance in the development of mammary neoplasms. Senescence is frequently associated with pituitary hyperplasia and development of adenomas in various strains of rats, whereas in humans the anterior lobe undergoes a slight or moderate involution, the pituitary weight decreases, and the incidence of adenomas is unchanged or slightly reduced (*102*). There are also wide differences in the morphology of the gland during development among various strains and among species. A description of the anatomy and physiology of the hypothalamus–pituitary system is beyond the scope of this review; therefore, only issues of essential importance for the comprehension of its interaction with the development of the mammary gland and its neoplasms will be attempted here. Despite interspecies differences, it is possible to establish a common pattern for this system (*98,102*). Of importance for the development of the mammary gland is the pars distalis, or anterior lobe of the hypophysis, which contains five categories of hormone-secreting cells: somatotropic cells, which secrete growth hormone (GH); lactotropic cells, secreting PRL; corticotropic cells, which secrete adrenocorticotropic hormone (ACTH) and its related peptides; thyrotropic cells, producing thyroid stimulating hormone (TSH); and gonadotropic cells, which secrete both luteinizing hormone (LH) and follicle stimulating hormone (FSH); sometimes called interstitial cell-stimulating hormone (ICSH) because of its action on the interstitial cells of both the ovary and the testis. The release of hormones from these cells occurs classically by extrusion of secretory granules. The hypothalamic component of the axis is not only important for control of the endocrine system and reproduction but also for the

Table 3. Hormonal role in normal mammary gland development

Hormone	Physiological role
Adrenocorticotropic hormone (ACTH)	Secretion and synthesis of cortisol and corticosterone by adrenal cortex
Follicle-stimulating hormone (FSH)	Stimulation of graafian follicles
Growth hormone (GH; somatotropin)	Overall body growth and stimulation of lactogenic activity
Luteinizing hormone (LH)	Stimulation of ovulation, estrogen secretion, formation of the corpora lutea, and progesterone secretion
Oxytocin	Stimulation of milk ejection by contracting of myoepithelial cells of mammae
Prolactin (mammotropin)	Stimulates mammae parenchyma, initiates and maintains milk production
Thyroxine	Maintenance of metabolic milieu, synergistic with insulin in mammary duct growth
Insulin	Stimulates mammary duct growth

From JT Stevens (personal communication).

Table 4. Comparison of the response to hormonal stimuli between humans and rats

Humans	Rats
The estrogen component of oral contraceptives leads to an increased incidence of thromboembolic disease (*103*)	The estrogen component of oral contraceptives does not lead to an increased incidence of thromboembolic disease (*103*)
Prolactin does not play a critical role in the nonpregnant human (*104*)	Prolactin plays a critical role in estrual animals (*105*)
Pituitary tumors are rarely seen in humans of either sex (*106*)	Administration of estradiol subchronically induces pituitary tumors (*107*)
Reproductive aging is the result of ovarian failure rather than neuroendocrine failure (*108*)	Reproductive aging is the result of neuroendocrine failure (*107*)
Ovarian failure is characterized by the depletion of the fixed supply of primary ovarian follicles (*109*)	Neuroendocrine failure is characterized by a decline of hypothalamic neurosecretory activity resulting in an inability to stimulate the preovulatory gonadotropin (LH) surge essential for ovulation (*110*)
At menopause, estrogen and progesterone secretion decline and pituitary gonadotropin secretion rises (*111*)	Loss of the preovulatory surge leads to an arrested ovarian follicular development, which results in persistent rather than cyclic estrogen secretion (*112*)
Rising gonadotropin (FSH, LH) secretion in postmenopausal women is evidence of intact hypothalamic function (*113*).	Exposure to endogenous estrogens occurs for a prolonged period of time creating an environment more favorable for mammary tumor development (*114*)

From JT Stevens (personal communication).

integration of numerous physiological functions, including thirst and water balance, body weight, body temperature, reactions to stress or emotions, sleep and arousal, and somatic reactions. Of critical interest are the feedback mechanisms exerted on the hypothalamus and factors released by the hypothalamus in mediating endocrine balance (Tables 3,4) (*103–114*).

PRL is capable of exerting a direct effect on the growth of the mammary parenchyma and epithelium (*79*). Gonadotropins have a significant influence on ovarian function; the secretion of FSH promotes the development of the Graafian follicles. LH is also required to bring the follicles to full maturity and to stimulate estrogen secretion. The corpus luteum, itself an endocrine gland, under the influence of LH secretes estrogen, progesterone, and inhibin (*69*). Finally, GH is also involved in mammary gland development and function. Although its exact mechanism of action is unclear, it directly stimulates duct growth in hypophysectomized–ovariectomized rats; however, the presence of estrogen is also necessary to evoke normal duct development. The prevailing metabolic condition of an individual animal or human may significantly influence mammary gland response to hormones. It has been demonstrated that estrogen and progesterone, when given with long-acting insulin, stimulate considerable mammary duct growth in hypophysectomized–gonadectomized rats (*72*). The growth-supporting effect of insulin can be enhanced by giving thyroxine. A synergistic effect of 11-desoxycorticosterone and estrogen on mammary duct growth has been observed in the ovariectomized virgin mouse. The various endocrine secretions influencing mammary gland development and function are summarized in Table 3. The maintenance of regular cycling events is important in rodents and humans for normal reproduction (*115*). There are remarkable differences in endocrine physiology between estrual and menstrual animals (Table 4) (*103–114*).

Spontaneous Rodent Mammary Tumors

Spontaneous mammary tumors are frequently observed in long term rodent studies (*116*). In mice, the development of spontaneous mammary tumors is linked to the infection of female mice with either an exogenous MMTV or a less virulent endogenous provirus. A third strain of MMTV transmitted through the milk and through the germ line has also been identified in the European mouse strain GR (*9–11*). The exogenous MMTV is an RNA virus first recognized to be transmitted through the milk of A and C3H strain mothers, the Bittner factor (*117*). DBA and RIII are also inbred strains of mice that harbor the highly oncogenic MMTV transmitted through the milk. Foster-nursed neonatal mice (i.e., C3Hf or DBAf) become free of the milk-transmitted MMTV although they retain the genetically transmitted MMTV, which induces mammary tumors late in life (*9–11*). In high-incidence strains of mice, tumors develop as a multistep process initiated in preneoplastic lesions, the hyperplastic alveolar nodules, which evolve from pregnancy-dependent to pregnancy-independent adenocarcinomas (*9*). Nulliparous mice develop a low incidence of mammary tumors. Out of a total of 1361 female B6Cf$_1$/CrlBR mice, only 5 adenomas (0.4%), 4 fibroadenomas (0.3%), 10 adenocarcinomas (0.9%), and 7 carcinomas (0.6%) developed by the end of a 24-month follow up (*118*). Some mammary tumors that develop in females of susceptible strains, such as C3H, A, DBA, 020, CBA, and certain sub-strains of Balb/c, are strongly hormone dependent in terms of their initiation. Multiple pregnancies enhance tumor development, and final tumorigenic response is greater in multiparous than in nulliparous animals. In RIII, BR6, DD, and GR mice, mammary tumors develop during the first pregnancy but they regress during lactation. In some strains of mice, the growth of mammary tumors is stimulated by chronic administration of estrogens, certain steroidal contraceptives, progesterone, PRL, and epidermal growth factor (EGF) (*9*), whereas hormone deprivation, induced by hypophysectomy, ovariectomy, ovariectomy-adrenalectomy, and syalidectomy suppress mammary carcinogenesis (*9*).

In the rat, the majority of spontaneously developed tumors, with the exception of leukemia, are neoplasms of endocrine organs or of organs under endocrine control. Spontaneous mammary tumors develop in females of various strains of rats such as August, Albany-Hooded, Copenhagen, Fisher, Lewis, Osborne-Mendel, Sprague-Dawley, Wistar, and Wistar/Furth (*9,45,116,119–121*). Mammary tumors are third in incidence among spontaneous tumors found in the Fisher 344 rat used in the National Cancer Institute/National Toxicology Program (NCI/NTP) carcinogenicity bioassays (*116*). They are predominantly benign tumors, i.e., fibroadenomas, fibromas, and more rarely adenomas. Malignant tumors such as adenocarcinomas are rare, although they are the most frequent tumors induced by chemical carcinogens (*24,47*). The development of spontaneous tumors varies as a function of strain, age, and endocrine influences. Mammary gland tumors develop in older females; they are more frequent in multiparous than in nulliparus rats. As in mice, hormone withdrawal inhibits tumor development in rats. Hormone supplementation, such as chronic administration of estrogens, increases the incidence of adenocarcinomas, whereas chronic administration of prolactin or of growth hormone stimulates benign tumor growth (*9*). The long latency period for spontaneous tumor development, up to 2 years in susceptible strains to develop a 50–70% tumor incidence, limits the usefulness of this model for experimental studies.

Experimental Genotoxic-induced Rodent Mammary Tumor Models

Chemically Induced Mammary Tumorigenesis

The potential of chemicals to induce cancer was recognized almost two centuries ago as an occupational disease when high incidence of skin cancer was linked to exposure to coal tar (*2*). Although it has not been proven that human breast cancer is caused by a given chemical or physical genotoxic agent, the human population is exposed to a large number of environmental chemicals such as polycyclic aromatic hydrocarbons, nitrosoureas, and aromatic amines, compounds that have been demonstrated to be carcinogenic in *in vivo* experimental animal models and have been shown to induce mutagenesis and neoplastic transformation of human breast epithelial cells in *in vitro* models (*122*). Although a specific etiologic agent or the conditions that might explain the initiation and progression of breast cancer in humans have not been identified, experimental animal models have proven to be useful tools for answering specific questions on the biology of mammary cancer relative to their validity to the human disease (*60*), as well as for assessing the risk for breast cancer posed by toxic chemicals (*7,37*). *In vivo* experimental animal models provide information not available in human populations: they are adequate for hazard identification, dose–response modeling, exposure assessment, and risk characterization, the four required steps for quantifying the estimated risk of cancer development associated with toxic chemical exposure (*7*). The use of experimental models of mammary carcinogenesis in risk assessment requires that the influence of ovarian, pituitary, and placental hormones, among others, as well as overall reproductive events, are taken into consideration because they are important modifiers of the susceptibility of the organ to neoplastic development (*9,41,44*).

Chemically induced mammary tumors develop by a multistep process. The initial

step is a biochemical lesion caused by the interaction of the carcinogen with cellular DNA. In this interaction the DNA is damaged; if the damage is not repaired efficiently, the result is a mutation, a chromosomal translocation, an inactivation of regulatory genes, or more subtle changes not well identified as yet. Neoplastic development requires that the lesion becomes fixed, aided by cell proliferation, and progresses to a third stage of autonomous growth, which results in cancer when the lesion acquires the capacity to invade and metastasize (*23*). Several carcinogens that induce mammary tumors in rodents have been identified and extensively studied for more than 50 years in mice and for more than 30 years in rats (*9*). In mice, mammary carcinomas have been induced with 3,4-benzopyrene, 3-methylcholanthrene (MCA), 1,2,5,6-dibenzanthracene, 7,12-dimethylbenz(*a*)anthracene (DMBA), and urethane in strains of low spontaneous mammary cancer incidence. Most of the mammary tumors induced in mice by chemical carcinogens are adenoacanthomas and type B adenocarcinomas. They develop after a relatively long period of time, and their induction requires multiple applications. Enhanced tumorigenicity has been obtained with prolonged hormonal stimulation; however, the hormone responsiveness of chemically induced mammary tumors in mice has not been as thoroughly studied as it has been in the rat (*9,23,60*).

In rats, DMBA, MCA, 2-acetylaminofluorene, 3,4-benzopyrene, ethylnitrosourea, butylnitrosourea, and *N*-methyl-*N*-nitrosourea (MNU) have been extensively used as mammary carcinogens, with DMBA and MNU used most frequently (*9,12–14, 23,41,60*). The majority of rat mammary carcinomas induced by either DMBA or MNU are hormone dependent. Maximal tumor incidence is elicited when the carcinogens are administered to young virgin females with an intact endocrine system (Table 5). These models of hormone-dependent tumors constitute useful tools for dissecting the multistep process of carcinogenesis and serve as a baseline for testing the carcinogenic potential of chemicals in risk assessment (*7*).

Radiation-induced Mammary Tumorigenesis

Ionizing radiation is probably the most widely acknowledged and studied human carcinogen (*47*). Exposure to radiation, either accidentally or for therapeutic reasons, has long been associated with a greater incidence of neoplasms, namely, hematopoietic, gonadal, and breast (*123*). The female breast is one of the tissues with the highest sensitivity to radiation carcinogenesis (*124*). Breast cancer developed in irradiated women shows a strong association with young age at the time of exposure, an association not observed in irradiated rodents (*123*) but similar to what has been observed in chemically induced mammary carcinogenesis in rats (*21,38,123*). Since controversy exists concerning the shape of the dose–response curve, the effects of fractionated irradiation, and the effect of low levels of radiation (*125*), animal studies are necessary to address these issues. The rat model has been widely used in this regard, mainly since the demonstration in the early 1950s that a single supralethal dose of X rays to female Holtzman rats (a Sprague-Dawley stock) maintained by temporary parabiosis induced an increased number of benign and malignant mammary tumors within 6 months of exposure (*47,126*). Sublethal doses of different types of radiation, including X rays and neutrons, have been shown to induce mammary tumor development, often within a year, with linear dose–effect relationships for neutrons over the total dose range and for X rays down to dose levels of 0.2 Gy (*127–129*). Irradiation of animals with fractionated doses of γ-radiation has resulted in linear-quadratic dose–response curves (*130*). Although most studies have used whole-body irradiation, localized irradiation also induces mammary tumors in the rat within the irradiated field. This effect also occurs in women, but reportedly not in several other animal species studied, e.g., mice, dogs, and guinea pigs (*127,128,131*). In rats, mammary carcinomas can be induced by whole-body or segmental radiation with either X rays, g rays or neutrons (*9,47*). Several studies utilizing a variety of fractionated irradiation protocols, i.e., at 12-hour intervals for 60 days (*130*), semiweekly for up to 16 weeks (*132*), and monthly for up to 10 months (*133*), have shown, in general, no increase in tumor latency, incidence, or total number of mammary tumors; no sparing or enhancing effect on mammary tumor development have been observed when compared with animals exposed to single doses of radiation. Some investigators, however, have reported an increased number of mammary carcinomas in animals receiving fractionated doses (*132*). Sprague-Dawley and Lewis rats are the most susceptible to radiation-induced tumorigenesis. AxC, Fisher, Long-Evans, and Wistar/Furth are also susceptible, but to a lesser degree. The mammary tumors developed by irradiated rats are, in general, hormone-dependent adenocarcinomas or fibroadenomas. The hormonal status of the female rat is of paramount importance in determining the outcome of irradiation of the mammary gland.

Table 5. 7,12-Dimethylbenz(*a*)anthracene (DMBA) or *N*-methyl-*N*-nitrosourea (MNU)-induced mammary carcinogenesis in female rats of different strains

				Mammary adenocarcinomas	
Carcinogen	Dose	Route	Rat strain	Incidence	No/rat
DMBA	20 mg/animal	ig	S-D	90	2.7
			W-F		2.6
			NSD		2.4
			Lew		2.3
			F		1.1
			ACI		0.4
			COP	0	0
	20 mg	ig	NSD		4.0
			COP	0	0
	1 mg	Topical	NSD	100	
			WF	100	
			F	40	
			ACI	25	
			COP	10	
	5 mg/animal	ip	NSD	100	1.2
			COP	0	0
MNU	50 mg/kg	iv	NSD	100	3.0
			F		1.2
			COP	0	0
	50 mg/kg	sc	NSD		2.4
			COP	10	0.1
	100 mg/kg	iv	NSD		3.9
			COP	0	0
	150 mg/kg	iv	NSD		4.8
			COP	0	0

ig, intragastric; ip, intraperitoneal; iv, intravenous; sc, subcutaneous; SD, Sprague-Dawley; W-F, Wistar-Furth; NSD, inbred S-D; Lew, Lewis; F, Fischer 344; ACI, A strain × C strain Irish: COP, Copenhagen. NSD rats received DMBA at a dose of 20 mg divided into four ig doses of 5 mg each and MNU at a dose of 100 mg/kg body weight divided into two iv doses of 50 mg/kg body weight each and at a dose of 150 mg/kg body weight divided into three iv doses of 50 mg/kg body weight each. Adapted from Russo et al. (*23*).

Ovariectomy completely prevents, and estrogen treatment enhances, radiation-induced mammary tumor formation (*47,134*). The latency period for tumor development is shortened and tumor incidence is increased considerably in estrogen-treated rats. The number of cribriform type adenocarcinomas (*47*) and the number of tumors per tumor-bearing rat (*134*) are also increased. Radiation and estrogens, namely 17β-estradiol (E_2) or diethylstilbestrol (DES), have been reported to exert either an additive (*135*) or synergistic effect (*136,137*). The effects of E_2 administration and irradiation on mammary tumorigenesis have been reported to be equal for hormone administration 1 week before or beginning 12 weeks after irradiation, but no additive effect has been observed when hormone administration was begun 24 weeks after irradiation (*135*). The amplification of radiation-induced mammary tumorigenesis by estrogens has been attributed by several investigators to the effects of this hormone on the pituitary, an interpretation supported by the observations that DES treatment of ACI rats and E_2 treatment of rats of three different strains, for example, result in increased incidence of pituitary tumors accompanied by marked increases in plasma PRL levels (*137–140*). The development of malignant mammary tumors in these rats appeared to be associated with the extent of increase in plasma PRL (*141*). Mammary tumor incidence and number or type of mammary tumors is not modified by irradiation during pregnancy, lactation, or postlactational regression in comparison with irradiation in the virginal state (*142*), in contrast to what has been reported in chemically induced mammary carcinogenesis (*21,38*). Furthermore, radiation-induced mammary tumors developed in rats do not exhibit the age dependency observed in women (*123*) or in chemically induced rat mammary carcinomas (*21,38*). They do not exhibit topographic selectivity in their development because they arise randomly in thoracic and abdomino-inguinal regions (*9,23*). Further studies are needed to clarify these differences in tumor incidence between radiation-induced and chemically induced mammary tumors in rats to validate this model for risk assessment. The reason for the differences in physiologic influences on the inductive action of chemicals and irradiation in rat mammary gland is not clear. It has been speculated that radiation-induced changes might occur in a specific stem cell population maintained throughout the reproductive life, while chemically induced changes depend upon the number and rate of turnover of other types of mammary gland cells (*19*).

Modifiers of the Susceptibility of the Mammary Gland to Neoplasia

Epidemiological Data

Breast cancer in women develops as the result of a combination of both internal and external factors. Increased risk is associated with nulliparity or late first full-term pregnancy, early menarche and late menopause, exposure to ionizing radiations at a young age, high socioeconomic status, and family history of breast or breast/ovarian cancer, including carriers of the newly identified *BRCA1* gene (*5,123,143*). Among the endogenous factors, early parity, defined as completion of a first full term pregnancy before age 24, has been identified as a protective factor (*5,22,144,145*). Very little is known, however, about the time of initiation of the carcinogenic process or what agent(s) cause it. The facts that early full-term pregnancy is protective (*5*), and that a higher incidence of mammary carcinomas has been reported to develop in women exposed to ionizing radiations at a young age (*123,146*) but not after pregnancy and lactation (*22,60*) strongly suggest that, in the human female, the period between menarche and first full-term pregnancy might be critical for the initiation of breast carcinogenesis. However, the precise time of initiation of the neoplastic process is not known. Regarding the site of origin of mammary neoplasms, there is evidence that tumors originate in the intralobular terminal ductal lobular unit (TDLU) or lobule type 1, the most undifferentiated structure present in the breast of nulliparous women (*23*). Correlative studies have shown an equivalence between the TEB of the young virgin rat mammary gland and the TDLU-lobule 1 of the woman's breast in their carcinogenic potential (*23,147–150*). Since human data are still insufficient for drawing a complete picture of the pathogenesis of breast cancer, adequate experimental systems mimicking the human disease are needed for addressing specific questions whose answer would allow one to elucidate (1) the influence of host factors on the initiation of the neoplastic process, (2) to determine whether the susceptibility of the human breast to undergo neoplastic transformation varies with age and reproductive history, and (3) to determine whether the susceptibility of the breast can be decreased through modifications of biological and physiological conditions of the host. We consider the model of rat mammary gland carcinogenesis to be the one that more closely fulfills the above conditions.

Use of Chemicals in Cancer Induction

Mammary tumors induced in rodents by chemical carcinogens have been widely studied and are useful models of mammary carcinogenesis (*16,17,24,26,28,47,52,151–154*). The objective of this review is to compare side by side what we know about the pathology of breast cancer in both humans and the rat model system to establish a solid basis for comparison and extrapolation of knowledge from animal to human. A comparative analysis will not only further our understanding of the biology of the human disease but will also highlight the gaps in knowledge that have to be filled in both systems. The two most widely used experimental systems of mammary tumorigenesis are the induction of rat mammary tumors by administration of either the indirect acting polycyclic hydrocarbon DMBA, given intragastrically (ig) to Sprague-Dawley rats (*28*) and the direct acting carcinogen MNU, given intravenously (iv) or subcutaneously (sc) to Sprague-Dawley or Fischer 344 rats, respectively (*155,156*). A single ig dose of DMBA 80–100 mg/kg body weight, induces tumors with latencies that generally range between 8 and 21 weeks. The final tumor incidence reaches 100% when the carcinogen is administered to intact virgin rats in their peak of maximal susceptibility, which in Sprague-Dawley rats occurs between the ages of 40–60 days of age (*38,157*). MNU, given in a single iv dose of 25 or 50 mg/kg body weight, yields tumors with similar latency and incidence (*157*). In a comparative study between the carcinogenic potential of DMBA administered ig at a dose of 20 mg and MNU, given iv at a dose of 50 mg/kg, it was demonstrated that both induce approximately equal tumor incidence and number of tumors per animal with approximately equal latency, but a somewhat greater percentage of MNU-induced tumors are histologically malignant (*32*).

For the study of modulating factors, especially when short-term studies are performed, it has been found that both tumor latency and tumor histological type are the most sensitive and reliable end points. Tumor latency is, in general, inversely related to carcinogen dose, whereas the overall tumor incidence and the number of malignant tumors per animal are directly related, especially when relatively early end points are used. No such relationship has been found for benign tumors (*32*). The number of tumors per rat or per group and the number or incidence of malignant tumors are additional end points useful in analysis of data. Comparison of data among laboratories requires strict standardization of the

experimental conditions because considerable variations in tumor incidence and latencies between laboratories and between experiments in the same laboratory are frequently seen (Table 6) (*157–162*).

Biological Bases of the Susceptibility of the Mammary Gland to Carcinogenesis

In every tissue, normal or abnormal, cell composition consists of a balance of three different cell populations: cycling cells, resting cells (cells in G_0), and dying cells (cell loss). In the mammary gland, these three cell populations can be identified through the study of the cell cycle and determination of the growth fraction and the rate of cell loss. The growth fraction refers to the fraction of cycling cells, while the rate of cell loss refers to the fraction of cells that die or migrate to other tissues. Both cell cycle time and the growth fraction determine the number of cells produced per unit of time, and the rate of cell loss determines the number of cells lost per unit of time. The growth of normal cells involves the net increase in cell number resulting from more cells being born than are dying. In differentiated tissue or in adult tissue in which growth has ceased, the number of cells produced per unit of time is equal to the number of cells that die. The higher susceptibility of the TEB to neoplastic transformation is attributed to the fact that the TEB is composed of an actively proliferating epithelium, as determined by the mitotic and DNA-labeling indices (Table 7) (*60*). These two indices are very high at the tip of both TEBs and TDs and decrease toward the ductal or proximal portion of the gland; however, TEBs have higher overall proliferative activity than TDs. Both mitotic index and DNA-LI are even lower in ABs and lobules (*17,19*). TEBs are also characterized for having the highest growth fraction, which progressively diminishes in the more differentiated ABs and lobules (*19*). By using these cell kinetic parameters, we have calculated the rate of cell loss in each one of the compartments of the mammary tree. Interestingly enough, the TEB is not only the structure with the highest proliferative ratio but also with the lowest percentage of cell loss in comparison with other parenchymal structures (Table 7). The rate of cell loss is very high in the lobular structures present in the mammary gland of parous rats. This clearly indicates that the TEB of the young virgin female is the truly proliferating structure of the gland that reaches a steady state only after acquiring full differentiation. The differences in proliferative activity and growth fraction observed between TEBs and the more differentiated structures of the mammary gland are also reflected in variations in the length of the cell cycle (Tc). Tc in TEBs of young virgin rats has an average length of 11 hr, increasing to 20.81 and 28.18 hr in TDs and ABs, respectively. Further mammary gland differentiation, as a consequence of aging and pregnancy, results in an even longer Tc, mainly due to a lengthening of the G_1 phase of the cell cycle (Table 7) (*19*). The length of Tc also varies according to the cell type and to the specific compartment in which each given cell type is located in. The shortest Tc is observed in intermediate cells located in TEBs, whereas it lengthens when the same cell type is located in ABs or lobules (*60*). These differences in the length of Tc are mainly due to differences in the length of the G_1 phase of the cell cycle, whereas all the other phases remain constant (*60*).

Mammary epithelial cells metabolize DMBA to polar metabolites with formation of epoxides that cause DNA damage. When dissociated mammary epithelial cells of young virgin and of parous animals, which basically represent the cells of the TEBs and of the lobules, respectively, are grown *in vitro*, they exhibit different rates of formation of polar metabolites. TEB cells produce more polar and less phenolic metabolites than lobular cells; this indicates that the TEB cells, in addition to their higher proliferative activity, are also producing more epoxides, which is manifested by a higher binding of DMBA to DNA. Autoradiographic studies performed *in vivo* confirm the observation that the greatest

Table 6. Histology of mammary tumors induced by 7,12-dimethylbenz(*a*)anthracene (DMBA) or *N*-methyl-*N*-nitrosourea (MNU) in female Sprague-Dawley rats

		Percent of mammary tumors			
		Adenocarcinoma			
Carcinogen	Dose	Invasive	Noninvasive	Fibroadenoma	Reference
DMBA	2.5 mg	10	54	30	(*157*)
		13	57	36	
	3.2 mg		52	48	(*158*)
			75	25	
	5.0 mg	6	80	14	(*157*)
		4	87	9	
	5.0 mg		98	2	(*159*)
	8.0 mg		34	66	(*160*)
	16.0 mg		64	26	
MNU	35–50 mg/kg		86–94	6–14	(*32*)
	20–30 mg/kg		71–80	20–29	
	10–15 mg/kg		42–59	41–58	
	50 mg/kg		94	6	(*161*)
	50 mg/kg × 2		93	7	(*13*)
	50 mg/kg × 2		97	3	(*162*)

Adapted from Russo et al. (*23*).

Table 7. Calculation of cell loss rate and birth rate in rat mammary gland

Rats	Structure	Growth fraction	Cell cycle	Labeling indices	S phase (hr)	Rate of cell birth	Terminal ducts	[(Ln 2 × 10)] / [TDT]	Rate of cell loss	Ø	Cell birth		Cell loss	Cell loss (%)
											Actual	Theoretic		
Young virgin	TEB	0.55	11.65	0.34	7.20	472.00	21.18	327.26	144.73	30.67	472.00	259.67	212.3	44.98
	TD	0.39	20.81	0.14	7.47	187.41	53.36	129.90	57.50	30.68	187.41	73.08	114.32	60.99
	AB	0.13	28.18	0.04	8.67	46.13	216.75	31.90	14.15	30.67	46.13	5.99	40.14	87.00
Old virgin	TD	0.190	18.75	0.077	7.60	101.35	98.70	70.22	31.13	30.71	101.33	19.25	82.07	81.02
	TD_R	0.054	20.57	0.020	7.60	26.31	380.00	18.24	8.07	30.67	26.31	1.42	24.87	99.70
	AB	0.030	30.75	0.008	8.20	9.75	1,025.00	6.25	3.50	35.89	9.75	0.29	9.45	96.99
Parous	TD	0.0097	23.92	0.003	7.40	4.05	2,466.66	2.81	1.23	30.37	4.05	0.03	4.01	99.00
	AB	0.0049	49.63	0.001	10.13	0.98	10,130.00	0.68	0.29	29.69	0.98	0.0048	0.97	98.90

TEB, terminal end buds; TD, terminal ducts; AB, alveolar buds; TD_R, terminal ducts in regression; rate of cell birth = labeling index/S phase length × 10^4; rate of cell loss = Ln 2 (0.693) × 10^4; TDT, total doubling time; Ø = rate of cell loss × 100/rate of cell birth; actual cell birth = growth fraction × 10^4/length of cell cycle; theoretic cell birth = growth fraction × 10^4/total doubling time; cell loss = actual cell birth minus theoretic cell birth; cell loss (%) = cell loss × 100/cell birth rate. Adapted from Russo and Russo (*60*).

uptake of [^{3}H]DMBA occurs in the nucleus of epithelial cells of TEBs and the lowest uptake in ABs and lobules, indicating that the highest DMBA–DNA binding is associated with the structure of the gland with the highest replicative properties (Table 8) (*22,163–165*). The ability of the cells to remove DMBA adducts from the DNA is an indication of their capability to repair the damage. TEB cells remove formed adducts less efficiently than lobular cells. This is attributed to the shorter G_1 phase of Tc and not to a lack of reparative enzymes (Table 8) (*22,164*).

These studies led us to conclude that the differentiation of the mammary gland modifies the following parameters: (1) gland structure; (2) cell kinetics, decreasing the growth fraction and lengthening the cell cycle, mainly the G_1 phase; (3) decreasing formation of polar metabolites and increasing phenolic metabolites; and (4) decreasing binding of the carcinogen (Table 8). All the parameters listed above affect the susceptibility of the mammary gland to carcinogenesis and should be taken into account when assessing chemicals for cancer risk.

Factors Influencing Tumorigenic Response

Influence of Mammary Gland Differentiation. Mammary cancer in experimental models is the result of the interaction of a carcinogen with the target organ, the mammary gland. This target, however, is extremely complex because the mammary gland does not respond to the carcinogen as a whole, but only specific structures within the gland are affected by given genotoxic agents. The knowledge of the architecture and cell kinetic characteristics of the mammary gland at the time of carcinogen administration constitutes a necessary initial step for understanding the pathogenesis of the disease. It is also required for distinguishing those changes induced by the carcinogen from changes reflecting normal gland development, especially when evaluating early tumorigenic response in short term studies (*15–19,22,55,145*).

The susceptibility of the mammary gland to DMBA- or MNU-induced carcinogenesis is strongly age dependent; it is maximal when the carcinogens are administered to virgin females between the ages of 40 to 60 days, that is, soon after vaginal opening and during early sexual maturity (*14,15,162*). Active organogenesis and a high rate of proliferation of the glandular epithelium are characteristics of this period in which there is also high DMBA activation (*17,20,162*). Its significance, however, is uncertain, because MNU, which is also most effective at this age, does not require activation (*13,166*). The incidence of DMBA-induced tumors reaches 100% when the carcinogen is administered to rats aged 30–55 days, but the highest number of tumors per animal is observed when the carcinogen is given to animals between the ages of 40 and 46 days, coincident with the period in which the mammary gland exhibits a high density of highly proliferating TEBs (*18,20,22*). This high susceptibility is attributed to the specific characteristics of the mammary gland that prevail during that period of life. Administration of DMBA to virgin rats induces the largest number of transformed foci when TEBs are decreasing in number due to their differentiation into ABs. These structures, instead of differentiating into ABs, become progressively larger due to epithelial proliferation, with multilayering, secondary lumen formation, and early papillary projections to the widened lumen. At this stage, transformed TEBs are called intraductal proliferations (IDPs) (Fig.

Table 8. Mammary gland degree of susceptibility

Parameters of susceptibility	High	Intermediate	Low
Morphological differentiation	TEB > TD > AB > LOB	TEB < TD > AB > LOB	TD < AB < LOB
Cell kinetics			
MI	7.03 ± 1.00	2.90 ± 2.30	0.09 ± 0.16
DNA-LI	34.40 ± 7.60	14.80 ± 4.70	0.30 ± 0.50
TC	9.93 ± 0.31	18.75 ± 0.99	49.63 ± 6.86
GF	0.55	0.19	0.0097
Lag phase in culture	none	24 hr	36 hr
No. of doublings in culture	3.04 ± 0.30	3.00 ± 0.14	1.50 ± 0.18
^{3}H-DMBA uptake (grains/nucleus)	6.80 ± 2.60	1.30 ± 0.80	0.70 ± 0.50
DMBA–DNA binding (μmol/DNA-P)	33	25	20
UDS, % of cells	20.0 ± 2.8	37.3 ± 3.06	62.5 ± 3.0
Adduct removal at 24 hr	15%	7%	25%
Carcinoma development	High	Intermediate	Low to none
Benign lesions development	Intermediate	High	Low to none

TEB, terminal end bud; TD, terminal duct; AB, alveolar bud; LOB, lobules; MI, mitotic index; DNA-LI, DNA labeling index; TC, length of the cell cycle (in hours); GF, = growth fraction; UDS, unscheduled DNA synthesis.

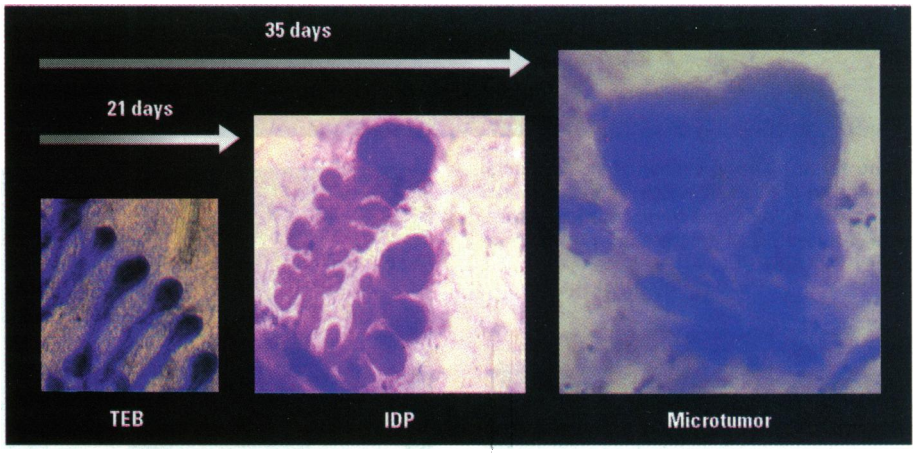

Figure 10. Terminal end buds (TEB; ×77) located in Zone C of the mammary gland of a 55 day-old virgin rat become enlarged and darkly stained by 21 days post-DMBA administration; they are called intraductal proliferations (IDP; ×79), which by 35 days post-treatment grow and coalesce to form microtumors that are not palpable (×129). Whole mount preparations, toluidine blue.

Table 9. Characteristics of the terminal structures of the rat mammary gland: control and experimental groups

Group no.	Structure	Size (μ)	No. cells/section	Percentage mitosis
1	TEB	103.0 ± 16.7	61.4 ± 26.7	7.03 ± 1.00
2	TD	69.6 ± 9.1	28.6 ± 9.9	2.90 ± 2.30
3	AB	31.9 ± 4.5	13.9 ± 2.2	0.09 ± 0.16
4	IDP	226.3 ± 49.0	110.0 ± 21.2	14.90 ± 3.30

Size in microns, number of cells/section and percent mitosis represent mean values ± standard deviation. Student's *t*-tests were done on all possible comparisons. For structure size, the following comparisons were significantly different ($p < 0.001$); group 1 vs. 2,3, and 4; group 2 vs. 3 and 4; and group 3 vs. 4. The percentages of mitotic figures for all groups were similarly significant ($p < 0.001$).

10, Table 9). Their confluence leads to the formation of microtumors, which can be classified histologically as intraductal carcinomas. These intraductal carcinomas progress to invasive carcinomas, developing various patterns such as cribriform, comedo, or papillary types (Fig. 10, 11, 12) (*15,24*). The histopathological appearance of these lesions is strikingly similar to the neoplasms developed in the human breast; a comparison between human and rat mammary tumors is shown in Table 10.

Even though TEB differentiation into AB is inhibited by carcinogen treatment, not all the TEBs present in the mammary gland at the time of DMBA administration progress to IDPs. Some TEBs still differentiate into ABs, but their number is always lower than that in control animals. Occasional lobular development is observed, although it is negligible. Some TEBs become smaller, exhibiting an atrophic appearance. At this stage, they are called terminal ducts (TDs). TDs are also susceptible to neoplastic transformation and are the main target of carcinogens in older animals (*40*). Those TEBs that were already differentiated into ABs and early lobular structures before DMBA administration do not develop carcinomas. Most of them either remain unmodified, undergo dilatation of the lumen and give rise to cystic structures, or exhibit epithelial proliferation and form tubular adenomas (Fig. 12,13). When old virgin females ranging in age from 180 to 330 days are inoculated with DMBA, they develop tubular adenomas that exhibit focal areas of malignant transformation, giving origin to well-differentiated adenocarcinomas with a tubular pattern. These lesions develop predominantly in the abdominal glands in which a higher incidence of tumors is observed in older animals. The observation that mammary carcinomas arise from undifferentiated structures of the gland, namely TEBs and TDs, and benign lesions such as adenomas, cysts, and fibroadenomas arise from structures that were more differentiated at the time of carcinogen administration indicates that the carcinogen requires an adequate structural target and the type of lesion induced is dependent upon the area of the mammary gland that the carcinogen affects. Thus, the more differentiated the structure at the time of carcinogen administration, the more benign and organized is the lesion that develops (Fig. 12) (*15,22,24,167*). The similarities observed between benign lesions developed in the human breast and the rodent mammary gland (Table 10) are indicative of a similarity in their pathogenetic pathways as well.

Cell of origin of rat mammary carcinomas. In the rat mammary gland parenchy-

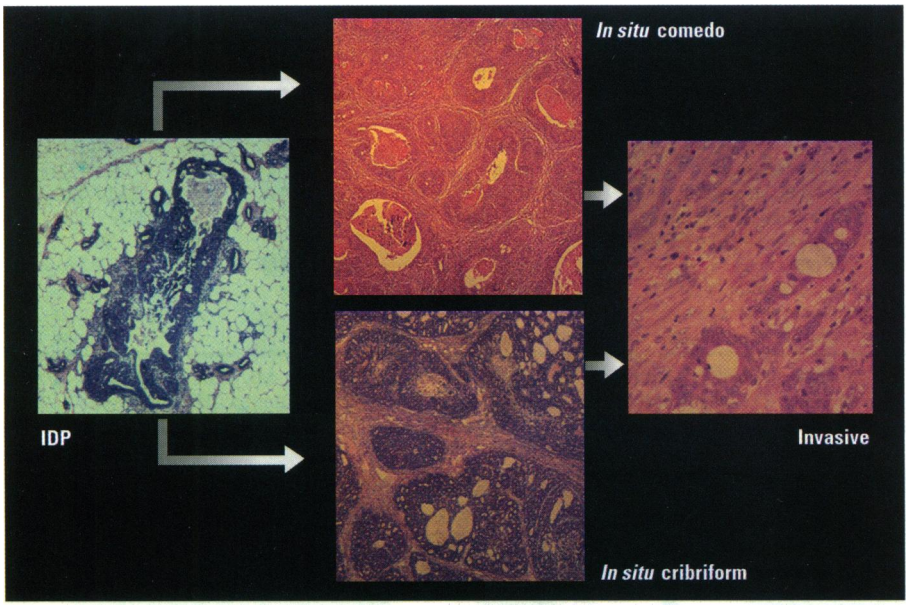

Figure 11. Histological section of an intraductal proliferation (IDP; ×110), which evolves to *in situ* carcinoma, either comedo or cribriform types. Both subtypes (×150) progress to invasive carcinoma (×150). H&E.

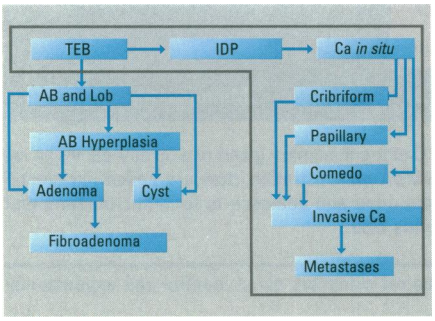

Figure 12. Pathogenetic pathways of benign and malignant lesions induced in the virgin rat mammary gland by DMBA. The undifferentiated terminal end buds (TEB) originate adenocarcinomas progressing from intraductal proliferation (IDP), to carcinoma (Ca) *in situ* to develop several subtypes of *in situ* and invasive carcinomas. More differentiated alveolar buds (AB) and lobules (Lob) originate benign lesions that appear later than carcinomas (*169*).

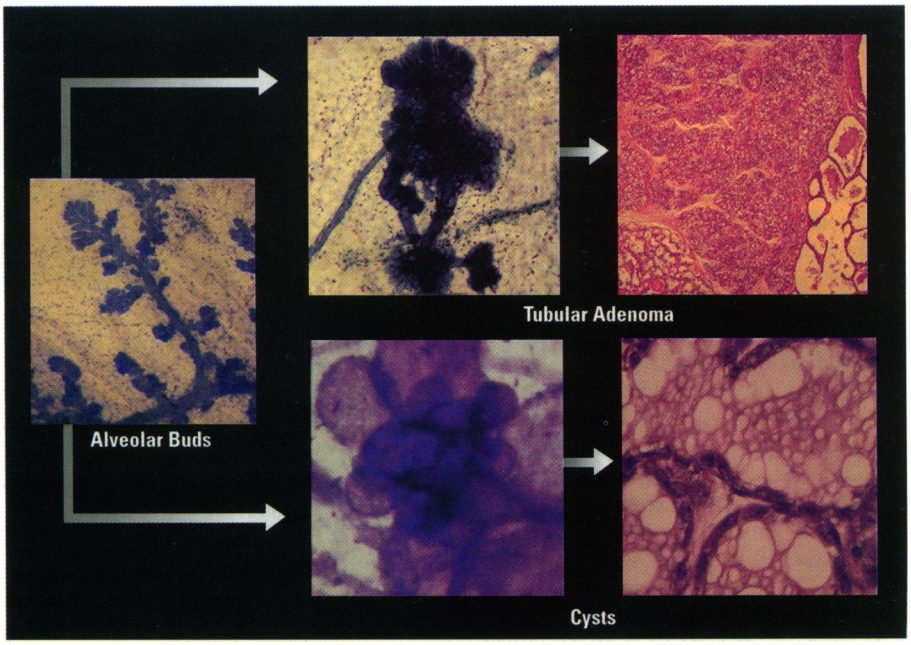

Figure 13. Evolution of benign lesions induced by DMBA in the mammary gland of virgin rats. Alveolar buds (×70) affected by the carcinogen develop tubular adenomas and cysts. Whole mounts, toluidine blue (×98); histological sections stained with H&E (tubular adenoma, ×160; cysts, ×179).

ma, three types of epithelial cells have been described (Fig. 3,5,6; Tables 11,12) (*56,57*). The distribution of cell populations in the mammary gland during carcinogenesis varies in TEBs and TDs, starting as early as at 24 hr post-DMBA administration, but no changes in cell composition occur in more differentiated structures such as ABs and lobules. The changes taking place in TEBs and TDs are limited to the dark-cell type, whose proportion decreases from 76% to 67%, and to the intermediate cell, whose proportion increases from 11% to 19%. Myoepithelial cells are unaffected. The trend is a progressive shift of cell population distribution, with a continuous decrease in dark cells and a concomitant increase in intermediate cells. By 14 days post-DMBA, intermediate cells constitute about 40% and 50% of the proliferative compartment in TEBs and TDs, respectively. At this time the morphological manifestations of tumorigenesis have started to become apparent. They consist of an increased number of epithelial cell layers, greater irregularity of the luminal border, and progressively larger intercellular spaces, which in some cases form secondary lumina. These features are indicative of the formation of an IDP. The basal lamina becomes distorted and the surrounding stroma becomes fibrotic and infiltrated by inflammatory cells, thus rendering the identification of myoepithelial cells more difficult. Between 21 and 40 days post-DMBA treatment, the descending curve of dark cells crosses over the progressively ascending curve of intermediate cells. By 40 days, the tumors display a cell distribution of greater than 65% intermediate cells with less than 35% dark cells. The proportion of intermediate cells continues to increase with tumor age, and at 70 days they represent greater than 75% of the total tumoral cell population. Dark cells, at this point, have been reduced to less than 20%, whereas myoepithelial cells remain at approximately 5%. The 100-day-old tumors are dominated by intermediate cells, which comprise nearly 90% of the total number of cells (Fig. 14) (*168*). The intermediate cells located in TEBs have a Tc lasting 13 hr. When the same cell type is located in more differentiated structures, such as ABs, they exhibit a lengthened Tc lasting 34 hr. These differences could explain the higher susceptibility of the intermediate cell of the TEBs to be affected by the carcinogen, which causes further expansion of the proliferative compartment of the intermediate cells and

Table 10. Classification of neoplastic and non-neoplastic lesions of the rat mammary gland with comparison to human lesions

Rat lesion	Corresponding human lesion
Benign epithelial neoplasms	Benign epithelial neoplasms
Adenoma (papillary, tubular, hyperplasia lactating)	Intraductal papilloma
	Lactating adenoma
	Adenoma of pregnancy
Malignant epithelial neoplasms	Malignant epithelial neoplasms
Ductal carcinoma	Ductal carcinoma
Non-invasive	Ductal carcinoma *in situ*
Cribriform	Cribriform
Comedo	Comedo
Solid	Solid
Papillary	Papillary
Invasive	Ductal carcinoma invasive
Cribriform	Cribriform
Comedo	Comedo
Solid	Papillary
Papillary (NOS)	Infiltrating ductal carcinoma (NOS)
NOS	Scirrhous
	Medullary
	Colloid
	Others
	Lobular carcinoma
	Lobular carcinoma *in situ*
	Infiltrating lobular carcinoma
	Paget's disease of the nipple
Stromal neoplasms	Stromal neoplasms
Benign	Benign
Fibroma	Fibroma
Malignant	Malignant
Fibrosarcoma	Fibrosarcoma
Epithelial-stroma neoplasms	Epithelial-stroma neoplasms
Benign	Fibroadenoma
Fibroadenoma	Cystosarcoma phyllodes, benign
Malignant	Carcinosarcoma
Carcinosarcoma	Cystosarcoma phyllodes, malignant
Non-neoplastic lesion	Non-neoplastic lesion
Normal lactating gland	Normal lactating gland
Lobular hyperpla	Lobular hyperpla of pregnancy
Lobular hyperplasia, not associated with pregnancy	
Cystic changes	Cystic changes
Ductal	Cystic dilatation of ducts
Lobular	Cystic dilatation of lobules
Epithelial hyperplasia	Epithelial hyperplasia without premalignant atypia
	Epithelial hyperplasia with premalignant atypia

NOS, not otherwise specified. From Russo et al. (*23*).

Table 11. Cell type distribution in the rat mammary gland

Structure	No. of cells counted	Cell type		
		Dark, (%)	Intermediate, (%)	Myoepithelial, (%)
TEB	2024	76.78 ± 8.6	10.97 ± 7.6	12.23 ± 3.3
TD + ducts	2731	75.64 ± 5.7	12.22 ± 4.8	12.12 ± 3.6
AB + lobules	2013	62.37 ± 13.3	20.74 ± 12.2	17.26 ± 6.8

Dark and intermediate cell types and myoepithelial cells were counted in terminal end buds (TEB), combined terminal ducts (TD) and ducts, and combined alveolar buds (AB) and lobules in toluidine blue-stained 1-µm sections of plastic embedded material. Values were expressed as the percentage of the total ± standard deviation. Student's *t*-tests were done on all possible comparisons; the differences were not significant.

Table 12. DNA labeling index in the cell types of the rat mammary gland

Structure	Cell type labeling index		
	Dark	Intermediate	Myoepithelial
TEB	14.25 ± 6.9	39.90 ± 20.3	16.20 ± 12.3
TD + Duct	3.95 ± 3.0	14.46 ± 11.5	5.16 ± 9.0
AB + Lob	0.49 ± 2.2	3.22 ± 6.9	2.25 ± 7.9

The DNA labeling index was determined as the percentage of cells incorporating ^{3}H-thymidine in autoradiographs of mammary gland sections. Values represent the mean DNA-LI ± standard deviation. TEB, terminal end buds; TD, terminal ducts; AB + Lob, alveolar buds and lobules combined.

depression in the dark cell population after initiation of the stimulus (*60*).

Role of the stroma in rat mammary carcinogenesis. In the preceding sections, we have shown that mammary carcinogenesis induced in Sprague-Dawley rats by administration of DMBA is the result of the interaction of the carcinogen with the TEB. When damaged by the carcinogen, epithelial cells of the TEB give origin to IDPs; these IDPs evolve to carcinomas. IDPs are morphologically distinguishable from TEBs by their size, which is more than twice that of TEBs and by the homogenous cell composition, which consists preponderantly of intermediate cells. The induction of IDPs is not a rare event. Within 3 weeks of DMBA administration, there are between 10 and 20 IDPs per mammary gland, and this number increases with time such that, by 6–10 weeks after treatment, there are approximately 30 lesions per gland and around 200 per animal. Although IDPs occur in large numbers following DMBA administration, the likelihood of any one IDP progressing to carcinoma in the intact mammary gland is lower than that because the maximal tumorigenic response rarely goes beyond 5 to 6 adenocarcinomas per animal. This phenomenon is attributed to the fact that there are two different types of IDPs. One type, initiated IDP (IDP [i]), increases in number steadily, with a concomitant decrease in the number of TEBs, and reaches a plateau by 60 days post-DMBA (Fig. 15). The IDP [i] is characterized by having a diameter larger than that of the TEB; it is composed of a greater number of epithelial cells and does not elicit a response in the surrounding stroma, remaining unchanged during the whole post-carcinogen observation period. A second type of IDP, initiated and promoted (IDP [i+p]), appears in the same proportion and at the same time as IDP [i], by 20–30 days post-DMBA (Fig. 15). They are also characterized by having a larger diameter and a greater cell number than TEBs; however, they elicit a marked stromal reaction, consisting of collagen deposition and infiltration by mast cells and lymphocytes. IDP [i+p] progresses to carcinoma *in situ* and to invasive carcinoma. The fact that not all the IDPs progress to carcinoma indicates that, although both IDP [i] and IDP [i+p] are preneoplastic lesions, there are factors that regulate the progression of initiated cells, which affect IDP [i] and IDP [i+p] differently (*169*).

The IDP [i+p] is surrounded by a number of mast cells three times higher than in both TEBs and IDP [i] (Fig. 16). This increase in mast cells is accompanied by an increase in lymphocytes, fibroblasts, collagen fibers, and proteoglycans. Mast cells are

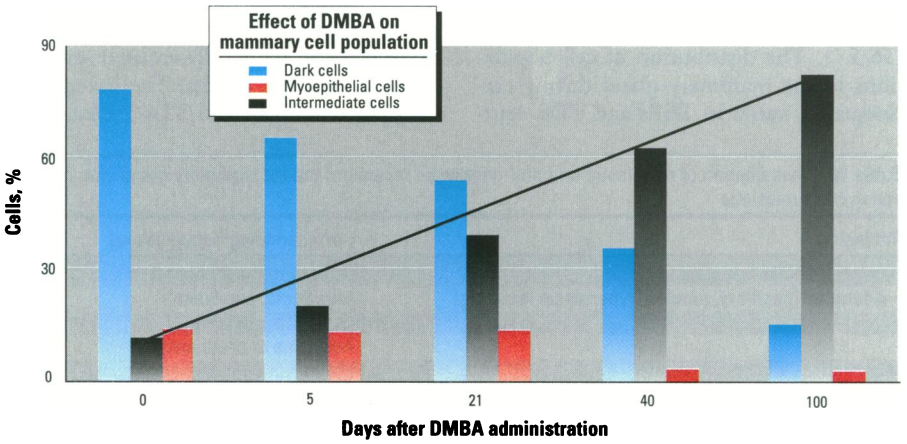

Figure 14. Effect of DMBA on mammary epithelial cell population. Dark cells, which predominate in the normal mammary gland ducts, become progressively replaced by intermediate cells. A slight reduction in number of myoepithelial cells is observed between 40 and 100 days post-DMBA administration.

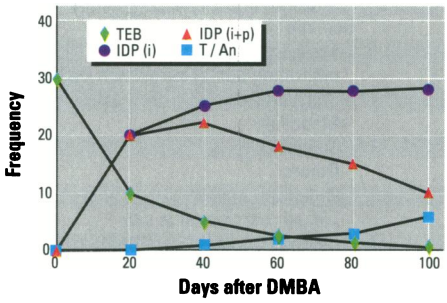

Figure 15. Emergence of microscopic preneoplastic lesions that were intraductal proliferations (IDP) initiated (i) and initiated and promoted (i+p), and total number of malignant tumors per animal (T/an). As the number of terminal end buds (TEB) decrease with time after DMBA administration, IDP reach a plateau by 40 days, and IDP (i+p) reach a peak at 40 days, decreasing in number as the number of tumor increases (*169*).

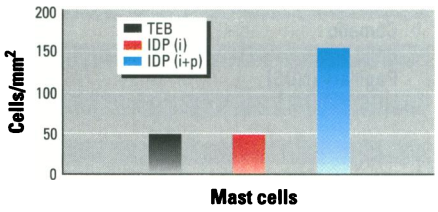

Figure 16. The number of mast cells per square millimeter is similar in terminal end buds (TEB) and in intraductal proliferations initiated (IDP [i]). A threefold increase is observed in IDP initated and promoted (IDP[i+p]).

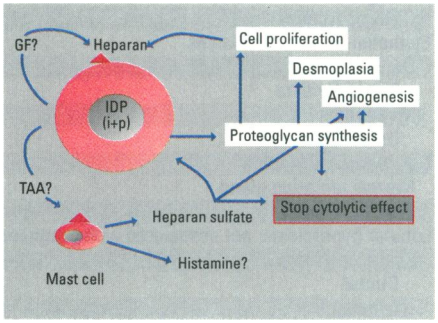

Figure 17. The transformed epithelial cells composing the intraductal proliferation (IDP) initiated and promoted (i+p) interact with stromal elements, attracting mast cells and stimulating local regulatory factors that result in increased synthesis of proteoglycans, which in turn affect cell proliferation, desmoplasia, and angiogenesis. GF, growth factor; TAA, tumor associated antigens. From Russo and Russo (*169*).

found in different parts of the body, and the mammary gland is not an exception. In the cytoplasm of mast cells, there are numerous granules measuring up to 0.8 µm in diameter, which stain metachromatically with toluidine blue or alcian blue (*170*). Mast cells degranulate, releasing histamine and heparin, a heparan sulphate that stimulates cell proliferation (Fig. 17) (*171*,*172*). Mast cell lysates or mast cell-conditioned medium stimulates locomotion of capillary endothelial cells *in vitro*, an effect that is attributed to heparin. It has been postulated that heparin or fragments of heparin on the surface of endothelial cells may selectively bind endothelial cell mitogens that are also angiogenic (Fig. 17) (*171*). An early change observed during the process of transformation is the synthesis of a large amount of proteoglycans by IDP [i+p], which is manifested by the deposition of an electron dense material on the cell surface of the epithelial cells (Fig. 18), and by an increased reactivity with alcian blue, pH 2.7, and periodic acid-Schiff (PAS). The increased synthetic activity is evidenced by an increase in uptake of ^{3}H-fucose and ^{3}H-glucosamine (Fig. 19). The number of cells absorbing these precursors is almost three times the number found in TEBs and IDP [i] (Fig. 19); this indicates that the initiated cells which are progressing to malignancy secrete heparan-type proteoglycans into the

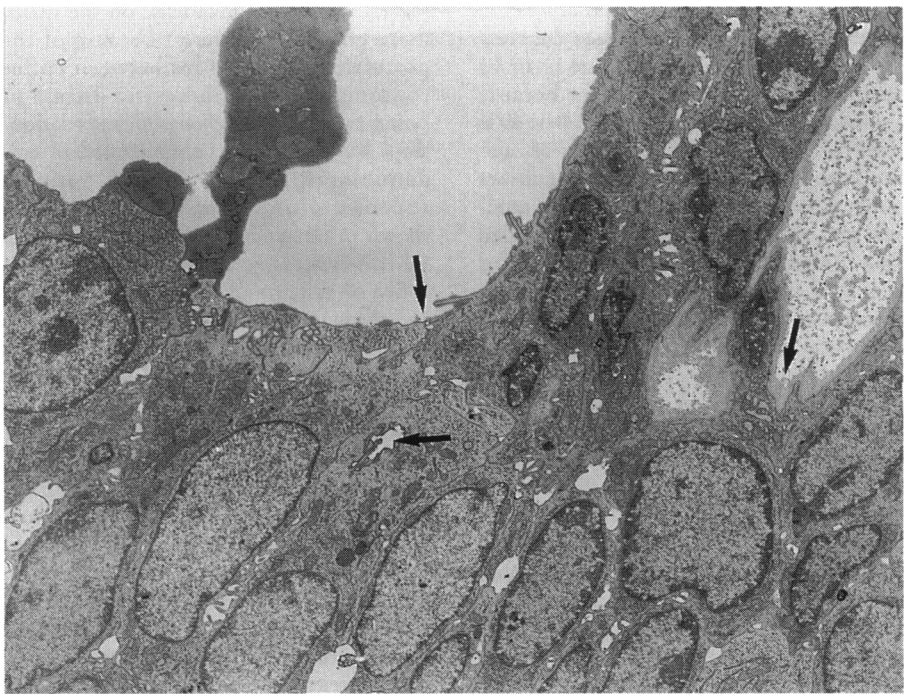

Figure 18. Ultrastructure of an intraductal proliferation showing accumulation of electron dense material (proteoglycans; arrows) on the luminal surface of the transformed epithelial cells and in newly formed intercellular spaces. Uranyl acetate-lead citrate (×1950).

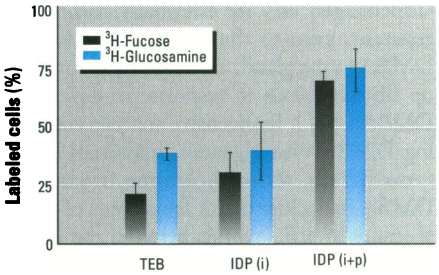

Figure 19. Percentage of cells labeled with ^{3}H-fucose and ^{3}H-glucosamine in terminal end buds (TEB), intraductal proliferation (IDP) initiated (i) and IDP initiated and promoted (i+p). From Russo and Russo (*169*).

stroma (Fig. 17) (*169*). It is not known whether these proteoglycans are influencing the response of the host by eliciting a higher mobilization of mast cells and inhibiting the cytotoxic effect of lymphocytes or by inducing angiogenesis, desmoplasia, and cell proliferation (Fig. 17). Some of these proteoglycans, such as heparan sulfate, act as receptors for growth factors, which in turn initiate an autocrine response (Fig. 17). Neoplastic transformation of cells dramatically alters proteoglycan synthesis in both the tumor and the surrounding tissues. This process is thought to stimulate tumorigenic growth by decreasing the adhesion of transformed cells to the extracellular matrix (*169*). Based on our own data and those reported in the literature, it is possible to postulate that the production of proteoglycans allows the IDP to progress to carcinoma *in situ* by stimulation of cell proliferation and by interference with an immune reaction against transformed cells (Fig. 17) (*169*).

Asynchrony in mammary gland development. For the induction of mammary carcinomas in the rat, the carcinogen is required to act on a specific compartment of the mammary gland, the TEB. Although these undifferentiated structures are present in all the mammary glands, tumor development does not occur as a random event in the six pairs of mammary glands. Tumor incidence in animals treated with the carcinogen between the ages of 20 and 180 days is greater in those glands located in the thoracic region, whereas glands located in the abdomino-inguinal area develop a lower number of tumors. In addition to differences in tumor incidence as a consequence of the topographic location of the gland, there are differences in tumor type that seem to vary with the age of the animal at the time of carcinogen treatment. Ductal and papillary adenocarcinomas are more frequent in both thoracic and abdominal glands of younger animals, whereas adenocarcinomas with a tubular pattern are found mostly in abdominal glands and in older animals (*60*). The pattern of development of the rat mammary gland, described as a branching of the parenchyma in ducts ending in TEBs that progressively differentiate into ABs and lobules, is common to the six pairs of mammary glands. However, this process does not occur simultaneously in all the glands, but differs depending upon the topographic location of each one of them. Individual structures, ie., TEBs, ABs, TDs, and lobules, appear similar in morphology in all the glands; however, their relative number and the general architecture of the organ vary notably from one pair of glands to another. The most notable ones are the thoracic mammary glands; each single gland is composed of two different layers separated by connective and muscular tissue. One of the layers is composed of more numerous ABs and small lobules, whereas the adjacent one is more extensive and contains thin long ducts ending in prominent TEBs. The abdominal glands have a markedly reduced number of TEBs, which are located exclusively in the most distal portion of the gland (zone C) (Fig. 2), whereas the middle and proximal portions A and B show a much more differentiated appearance. The difference in number of TEBs in thoracic versus abdominal mammary glands is significant. The number of TEBs decreases progressively with aging. The reduction is proportional in all the glands, and it is mostly due to the regression of TEBs to TDs or to their differentiation into ABs and lobules. The higher incidence in ductal carcinomas observed in thoracic glands is attributed to the difference in degree of development of the undifferentiated layer of this gland in comparison with the glands located in other topographic areas (*40*).

Genetic influences. Genetic differences among individuals may affect their susceptibility to the carcinogenic effect of chemicals. Inheritance may also predispose an individual to develop certain specific types of cancer (*173*). These influences have been carefully examined in rodent experimental animal models (*174,175*). In carefully designed experiments, it has been demonstrated that the susceptibility of rats to the chemical carcinogens 2-acetylaminofluorene (AAF), DMBA, and MNU is genetically determined (*174,175*). Isaacs (*175*) demonstrated that Buffalo, Lewis, Wistar/Furth, and inbred Sprague-Dawley rats, all strains of Wistar genetic background, are highly susceptible to chemically induced carcinogenesis, whereas the non-Wistar derived strains (Fischer, August, ACI, and Copenhagen) are of low susceptibility. However, there are exceptions to this rule: the Wistar-derived inbred WN strain is of low susceptibility and the non-Wistar derived Osborne-Mendel is highly susceptible. Of the commonly used strains, Sprague-Dawley and Wistar-Furth are the most susceptible, and Fischer 344 and ACI rats show intermediate susceptibility.

Copenhagen rats are essentially completely resistant, even to the direct application of DMBA to the gland, although they do develop fibrosarcomas in response to parenteral DMBA (*174*). In extensive analyses comparing DMBA tumorigenesis, mammary gland growth rate, serum hormone levels, and DMBA toxicokinetics in female rats of several strains and F_1 hybrids between the strains (*174,175*), no major differences that correlated with susceptibility to tumorigenesis were found because both susceptible and resistant strains developed similar percentages of malignant changes (60% in resistant, 80% in susceptible). However, macroscopically detectable tumors developed in 70% of susceptible glands and 10% of resistant glands. These findings have been confirmed by transplantation experiments between resistant or susceptible strains into F_1 hybrids, between the two strains, and by direct exposure to DMBA. These observations indicate that genetic factors govern the progression from microscopic to macroscopic tumor rather than from normal to histologically malignant epithelium. Furthermore, these observations demonstrate that resistant rats possess a dominant suppressor allele for the gene governing susceptibility. In contrast, tumor induction by diethylstilbestrol (DES) is demonstrable in the ACI strain but not in Sprague-Dawley rats, although a cocarcinogenic effect of DES with DMBA can be shown in the Sprague-Dawley strain (*176*). Both malignant and benign tumors are increased by the combined treatment, but there is a relatively greater increase in benign tumors. The response to DES of target organs other than the mammary gland is also different in these two strains of rats, but the mechanisms are not known (*177*).

Dietary influences. Carcinogenesis can be modified by nutrients and other dietary constituents, by other chemicals, and by endocrine alterations. Excessive fat intake affects mammary tumorigenesis by reducing tumor latency, increasing tumor multiplicity, and increasing the fraction of histologically malignant tumors (*81,86,158, 178,179*). To yield valid results, rats fed high fat diets must be compared with rats fed diets that supply sufficient fat for normal growth and development, at least 4 to 5% fat by weight, and sufficient essential fatty acids. The control diet must be equivalent in all nutrient-to-calorie-ratios to the high fat diet. The two groups of rats must show comparable weight gain or controls must be included to permit evaluation of caloric and specific fat effects on tumorigenesis because tumor incidence is increased with increased caloric consumption (*180,181*). Studies of the mechanisms by which fat may act have been reviewed extensively in recent publications (*157,176*). No mechanism other than increased caloric intake has been convincingly demonstrated to contribute to or be responsible for the effect of fat because reduction of caloric intake by 20, 30 or 40% (*180–182*), even with a percentage of calories from fat held at a high level, increases tumor latency and reduces incidence, number, and size of tumors. However, added caloric intake and weight gain by rats fed high fat diets do not account for correlation of increased tumorigenesis with type, as well as amount, of fat (*157,166,183–187*) or for results of paired-feeding studies (*184*).

In DMBA-treated rats in which the energy intake from fat (corn oil) had been doubled from 25 to 48% of calories (from 10.5 to 24.6% by weight) for the 4 weeks between weaning and DMBA administration, mammary tumor incidence increased significantly; the odds ratio for carcinoma increased by a factor of 1.6 and for any tumor by a factor of 5. Body weight and caloric intake over the 4-week period were not affected by dietary fat content, provided that the protein content was adequate for growth. This result and studies using lard in the place of corn oil (*157,184–186*) confirmed an effect of fat on the initiation of tumorigenesis. After DMBA was given, all rats were fed the same diet with 24% of calories as fat; a positive effect of caloric intake on tumor incidence was found to be independent of dietary fat content. The high fat diets that enhance tumorigenesis do not alter significantly: (1) toxicokinetics of DMBA (*188*); (2) blood levels of PRL, 17β-estradiol, progesterone, or LH or the patterns of hormone secretion through the estrous cycle (*185,186,189*); or (3) [^{3}H]thymidine labeling of mammary gland epithelial DNA before or after carcinogen exposure (*190*). Although induction of both hormone-dependent and hormone-independent tumors responds to dietary fat content (*185,186*), high fat diets do not increase the growth rate of tumors as measured by palpation (*157,178*).

The influence of caloric use by voluntary or involuntary exercise is under investigation. Unfortunately, results are variable and show both increased and decreased tumorigenesis in exercised rats (*190*). Standardization of methods with measurement of energy intake and expenditure and of body composition are needed to permit comparison and interpretation of results. There is renewed interest in the effect of omega-3 fatty acids on growth and metastasis of transplanted, as well as induced, mammary tumors in rats. However, the results are not entirely consistent within or among laboratories, probably in part because acceptance of the diets by rats varies (*191*).

The influence of caffeine on the mammary gland is of interest because of the postulated relationships between coffee consumption and fibrocystic disease or breast cancer, although significant relationships have not been demonstrated in epidemiologic studies. Highly variable responses to coffee and caffeine have been shown in rats and mice (*159,192,193*). In DMBA-treated Sprague-Dawley rats given coffee or caffeine in drinking water before and during DMBA initiation of mammary tumorigenesis, tumor multiplicity, but not tumor incidence or tumor latency, was reduced in comparison with rats given DMBA alone. When coffee or caffeine were given after DMBA administration, there was no consistent effect on tumorigenesis (*192*). Results were the same when rats were fed a purified diet that contained 5 or 20% fat (by weight) (*193*). In mice given similar amounts of coffee or caffeine, DMBA-induced or murine mammary tumor virus-induced tumor multiplicity was increased but other parameters of tumorigenesis were not affected. Caffeine increased mammary gland development in mice, apparently by increasing the response to trophic hormones (*159*).

Epidemiologic studies strongly indicate that alcohol intake is a risk factor for breast cancer (*194,195*). The increased risk is 1.5- to 3-fold, depending upon the population studied and the amount of alcohol consumed. In experimental animals, alcohol may have an inhibitory effect on the liver metabolism of certain compounds such as nitrosamines that are carcinogenic in a variety of sites. In rats treated with MNU, which does not require metabolic activation, daily doses of ethanol (5 mg/kg body weight) were associated with increased numbers of malignant tumors but not significant alteration of tumor latency or incidence (*195*).

Impact of Hormones and Growth Factors in Mammary Carcinogenesis

The role of hormones on the development of the mammary gland has been the subject of numerous studies which over the years have generated a bibliography too voluminous to be comprehensively cited here (*66,101,196*). Despite of the amount of knowledge available, the subject is continuously under scrutiny and revision as new growth factors are discovered or characterized and hormone receptors are reassessed in their locations and functions. Nevertheless, classic knowledge has established that mammary gland development occurs only in the presence of a functional ovary. The pubertal development of the female rat, therefore, has been divided, based on ovarian morphology

and serum gonadotropin levels, into the following phases: 1) neonatal period, from birth to day 7; 2) infantile period, from day 8 to day 21; 3) juvenile period, between 22 and 32 days; and 4) peripubertal period, encompassing the next 3 days of life (*68*). The ovary, in turn, depends on pituitary gonadotropins for its development and function; receptors for LH and FSH actively bind these hormones as early as the infantile period, although the stimulated secretion consists of androgens rather than estrogens. Pituitary FSH interacts with GH and PRL in modulating ovarian steroidogenesis, a function that is also influenced by epinephrine, which is secreted by the adrenal medulla (*68*). The ovary also secretes inhibin and activin, non-steroidal glycoprotein hormones that feed back to the pituitary, specifically in an FSH-release suppression function (*70*). The response of the mammary gland to these complex hormonal interactions results in developmental changes that permanently modify the architecture and the biologic characteristics of the gland. The mammary gland, in turn, responds selectively to given hormonal stimuli depending upon specific topographic differences in gland development, which modulate the expression of either cell proliferation or differentiation, as described above.

Ovarian Steroid Hormones and Growth Factors

The ovary secretes three main classes of steroid hormones (estrogens, androgens, and progestins). Their relative and absolute amounts change periodically during the phases of the menstrual cycle in humans and the estrous cycle in rodents. The three main natural estrogenic hormones synthesized by ovarian follicles are estradiol, estrone, and estriol. Estrogens are critically involved in mammary gland development and are also essential for eliciting a tumorigenic response with chemical or physical carcinogens (*47,197*). Estrogens act on mammary epithelial cells through three different mechanisms: a direct receptor-mediated effect, a mammary stroma-mediated effect, and an *in vivo* stimulation of pituitary PRL levels, which in turn, stimulates lobuloalveolar development in the mammary gland (*67,76*). Estradiol is the most potent and abundant estrogenic hormone and is responsible for the beginning of estrus. It is oxidized in the liver to estrone, which in turn can be hydroxylated to estriol. Estriol decreases the effects of estradiol, thus acting as a partial antagonist. Continuous administration of supraphysiological doses of estrogens, either by implantation of pellets or subcutaneous injection, induces a high percentage of rat mammary tumors, which are predominantly adenocarcinomas (*154*), whereas low doses given over long periods induce fibroadenomas (*198*). The tumorigenic effects of estrogens are dependent on a functional pituitary gland because they are ineffective in hypophysectomized rats (*101,199*). Secondary changes in the pituitary gland have been found in rats after prolonged administration of estrogen. Treatment of female F344 rats with DES causes a 10 to 16-fold enlargement of pituitary glands, an increase in number of hyperplastic PRL-secreting cells that exhibit hyperplasia of the rough endoplasmic reticulum, and decreased secretory granules. Estrogens stimulate the growth of DMBA- and MCA-induced mammary tumors when administered at low doses; however, the development and growth of these adenocarcinomas are inhibited by high doses of 17β-estradiol, estriol, estrone, and DES. This biphasic effect might be explained by the stimulation of PRL secretion by low levels of estrogens, and the inhibition of PRL by large doses (*197*). Ovariectomy either prior to or very soon after MCA or DMBA administration suppresses rat mammary carcinoma development; it also inhibits tumor growth or causes tumor regression in rats already bearing tumors. Reactivation of tumor growth in ovariectomized rats can be accomplished by administering moderate levels of 17β-estradiol or DES (*101*). The combination of estrogen and PRL is essential for the neoplastic transformation of the mammary epithelium, because no tumors are induced by administration of estrogens following hypophysectomy. Hypophysectomy alone, on the other hand, does cause tumor regression (*197*).

Progesterone, one of the main hormones produced by the corpus luteum (*200*), when implanted in female rats increases the frequency of both MCA- and DMBA-induced mammary tumors in either intact or ovariectomized rats but does not do so in ovariectomized–adrenalectomized rats (*25,27,64,65,201–205*). Chronic administration of progesterone to neonatally androgenized rats at varying times after DMBA treatment causes a marked increase in development and growth of mammary carcinomas (*101*); however, moderate to high doses of this hormone, in combination with high doses of estrogen, inhibit the growth of DMBA-induced rat carcinoma (*28*). The latter treatment causes intense hyperplasia in the normal rat mammae and also causes ovarian atrophy. It is curious that a moderate dose level of progesterone, by itself a mammary tumorigen, coupled with estrogen at high dose levels, provides a therapeutic hormonal milieu considerably more effective than treatment with estrogen alone at high doses (*12,28,101*). It is important that moderate doses of progesterone or estrogen (natural or synthetic) can enhance the growth of polycyclic aromatic hydrocarbon-induced rat mammary carcinomas.

Inhibins are heterodimeric growth factors, members of the transforming growth factor β (TGF-β) superfamily. These glycoprotein hormones are produced in the gonads; their known function is a feedback mechanism to the pituitary for inhibiting the production and release of FSH (*69,70,85*). The granulosa cells of the ovary in most species and human luteal cells have been identified as the sites of inhibin synthesis in the female. Inhibin mRNA has been detected in extragonadal tissues; recently its synthesis by the rat mammary epithelium in response to human chorionic gonadotropin (hCG) treatment and during pregnancy has been reported (*69*). These findings and the observation that inhibin-deficient transgenic mice develop gonadal tumors (*206*) indicate that this peptide has a tumor suppressor activity, in addition to autocrine or paracrine growth and differentiation modulator functions.

Pituitary Hormones

Two endocrine bodies, the hypothalamus and the pituitary gland, have a role in the control of prolactin secretion. The anterior pituitary is able to synthesize and release large quantities of prolactin upon the destruction of hypothalamic connections. The hypothalamus inhibits prolactin secretion via production of prolactin-inhibiting factor (PIF), which is in turn controlled by the amount of catecholamines released from nerve endings in the hypothalamus. Rising catecholamine concentrations result in release of PIF and reduced release of prolactin by the pituitary. A decrease in catecholamine release produces the opposite effect. Estrogen acts on the pituitary gland to increase prolactin release and on the hypothalamus to decrease PIF, thus increasing prolactin secretion by dual mechanisms. In addition, the thyroid hormones, acting directly on the pituitary, also increase prolactin secretion (*199*). The influence of prolactin on the induction and growth of spontaneous or oncogene-induced mammary neoplasia in rats and mice is well established (*207–211*). Grafting of multiple pituitary isographs to mice significantly increases the incidence of mammary tumors. This same procedure used on nulliparous and multiparous rats greatly increases this tumor incidence over that of the nongrafted controls, regardless of parity status (*212*). In the rat,

production of median-eminence hypothalamic lesions in females results in a significant increase in spontaneous mammary neoplasia. Such lesions in the rat increase prolactin secretion and decrease or prevent secretion of other anterior pituitary hormones. All of these effects indicate that prolactin is the major pituitary hormone in spontaneous mammary oncogenesis and that an endocrine imbalance can be tumorigenic in the rat and the mouse. There is a direct correlation between prolactin levels in serum and the genetically influenced susceptibility of several rat strains to induced mammary tumorigenesis (*213*). The following treatments and physiological states cause hyperprolactinemia in female rats bearing DMBA- or MCA-induced mammary tumors and significantly increase growth of these neoplasms: adrenalectomy; pregnancy; pseudopregnancy; pituitary homografts; pituitary tumors; hypothalamic lesions; hypothalamic implants of steroids; certain neuroendocrine-influencing drugs such as reserpine, perphenazine, and haloperidol; and high dietary fat. Hypoprolactinemia induced by a number of chemicals (e.g., ergot alkaloids, cyclic imide derivatives, lysergic acid, ergoline derivatives, L-dopa, pargyline, antirat prolactin serum) causes significant decreases in growth of induced tumors (*101*). The reduction in serum prolactin levels, which is generally dose related, is associated with an increase in hypothalamic catecholamines and PIF (*214,215*). An excess of prolactin is often mitogenic in normal rodent mammary tissue and appears to be a critical regulatory hormone for controlling mitotic activity of mammary epithelium (i.e., deficiency causes hypoplastic epithelium and excess results in mammary hyperplasia). Prolactin-induced changes in mammary mitotic activity might influence the susceptibility of the epithelium to chemical, physical, and viral oncogenic agents. Clearly, fluctuations in levels of prolactin influence the growth of mammary tumors in the rat; however, there does not appear to be a correlation between normal range levels of serum prolactin and either fast- or slow-growing mammary tumors (*213*). It is possible that variations in oncogene-induced growth might be the result of the sensitivity of mammary tumor cells to prolactin; thus, small changes in prolactin secretion, not detected by serum analysis, could markedly influence tumor cell proliferation. That prolactin is also the key hormone in mouse mammary neoplasia is supported by the observation that treatment with ovine prolactin or reserpine or the induction of hypothalamic lesions greatly increases the incidence of such tumors in this species. In many strains of mice, prolonged above-normal prolactin secretion invariably accompanies an increase in mammary tumors. Treatment of nulliparous C3H/HeJ mice with either 2-bromo-α-ergocryptine or 6-methyl-8-β-ergoline-acetonitrile, both prolactin-suppressing drugs, virtually prevents or sharply reduces mammary tumorigenesis. It appears that prolactin is not only an important hormonal stimulant of mammary tumorigenesis but is essential in the neoplastic transformation of the mouse mammary gland (*101,213*). Prolactin is the principal pituitary hormone in the development, maintenance, and transformation of hyperplastic alveolar nodules (HAN). Prolactin secretion-suppressing substances reduce the number of HANs, as well as the number of mammary tumors. Although prolactin by itself plays an important role in induction and growth of mammary tumors in rodents, it may also act synergistically with other hormones (e.g., estrogens) or by possibly reacting with other host factors. The simultaneous development of mammary tumors and neoplasia of the pituitary has been observed. Pituitary tumors have been known to develop directly as effects of estrogen on pituitary luteotrophs (*216*) and indirectly by injury of dopaminergic tuberoinfundibular neurons in the basal hypothalamus, which secrete prolactin-inhibiting factor (*217*). The uninhibited activity of these neurons with further stimulation by estrogen is the basis of the pituitary tumor development. The high spontaneous pituitary neoplasia in various rat strains is considered to be, at least in part, due to hyperestrogenism in aging females (*218*). The estrogen-induced and spontaneous pituitary tumors have been demonstrated to be primarily prolactin secreting. Data seem to substantiate that estrogens are mammary carcinogens in rodents because of their stimulatory effects on prolactin secretion. However, the role of prolactin in the development of breast cancer in women is uncertain (*219*).

Placental Hormones and Growth Factors

The reproductive process, since its initiation, is deeply dependent of hormonal and neural factors. The maternal corpus luteum, which is instrumental in the preparation of the endometrium for implantation, is in turn rescued by the luteotropic hormone chorionic gonadotropin secreted by the primitive trophoblast of the blastocyst within hours of implantation. In women, hCG stimulates the corpus luteum to synthesize progesterone, 17-hydroxyprogesterone, estradiol, inhibin, and relaxin (*98*). hCG also stimulates the synthesis of inhibin in the rat corpus luteum (*69*). Relaxin plays a major role in the maintenance of early pregnacy because it causes the relaxation of the myometrium and, in conjunction with progesterone, reduces spontaneous uterine activity (*220*). The corpus luteum constitutes the major source of progestational steroids until the ninth week of gestation when the placenta becomes the sole source of these hormones, as demonstrated by the lack of effect of ovariectomy after the ninth week on the progression of pregnancy. The placenta has evolved in mammals as an efficient mechanism for transporting nutrients to the fetus, excreting waste products into the maternal blood stream, and influencing maternal physiology through the newly secreted placental and fetal hormones. In humans, the placenta becomes fully developed by the end of the first trimester of pregnancy. The functional unit is the chorionic villus, which is composed of an outer layer of trophoblast, the syncytiotrophoblast, and an inner layer, the cytotrophoblast, both arranged around a core of loose connnective tissue traversed by numerous fetal capillaries. The syncytiotrophoblast synthesizes progesterone through hydroxylation and side-chain cleavage of cholesterol. The syncytiotrophoblast also produces and secretes hCG and human placental lactogen (hPL). The cytotrophoblast is the source of several neuropeptides first discovered in the brain, such as gonadotropin releasing hormone (GnRH), thyrotropin releasing hormone (TRH), somatostatin, corticotropin releasing factor (CRF), and propiomelanocortin. It also synthesizes the gonadal peptide inhibin (*220*). Chorionic gonadotropin (CG) is a polypeptide hormone composed of α and β subunits. The α subunit is identical to that of pituitary gonadotropins, whereas the β subunit differs in amino acid sequence (*221*). The most widely known action of CG is the maintenance of the corpus luteus during pregnancy. The action of CG is identical to that of the pituitary gonadotropin LH, with a small degree of FSH activity (*69,222*). CG produced by the placenta of rats, mice, and hamsters is structurally similar to human CG (*223–226*).

Insulin

Insulin is a hormone synthesized by the β cells of the islets of Langerhans, the endocrine portion of the pancreas (*98*). It is produced in the form of a biosynthetic precursor of higher molecular weight, proinsulin. The structure of insulin has been relatively stable throughout evolution, and most mammalian insulins have similar biological potencies in all species (*98*). Glucose is the major stimulus for both the synthesis and secretion of insulin. Insulin, in turn, regulates glucose entry in most tissues (*227*). It exerts an acute metabolic effect, i.e., glucose tolerance; it also elicits growth responses and evokes late or long-term biological effects

(*228*). The development, function, and even tumorigenesis of the mammary gland depend on insulin for expression. The mammary gland primordium of the 17-day rat embryo grows and penetrates the mesenchyme in response to insulin (*44*). Insulin plays an essential role in the expression of terminal differentiation of the mammary epithelium. Physiological levels of insulin, in the presence of cortisol and prolactin, induce the synthesis of casein by mouse mammary explants *in vitro* and promote the induction of α-lactalbumin in cultured rat mammary tissue, even in the absence of glucose in the medium (*228*). Insulin or a glucose solution administered to rats bearing DMBA-induced mammary carcinomas significantly increases tumor growth; when these two substances are administered in combination, the treatment produces a much larger tumor growth response (*229,230*). Alloxan diabetes in tumor-bearing rats induces a significant regression of mammary tumors, quantitatively resembling the regression observed with ovariectomy or hypophysectomy. Administration of insulin activates mammary carcinoma growth in intact rats and reactivates growth in regressing tumors in hypophysectomized rats (*231,232*). Studies have shown that insulin plays an important role in regulating tumor estrogen receptors (*233*). Insulin has a direct growth-promoting effect on rat mammary carcinoma cells *in vitro* (*232*).

Androgenic Steroids

Androgenic steroids can be classified in two categories: adrenal androgens (androstenedione, 11β-hydroxyandrostenedione, dehydroepiandrosterone, and dehydroepiandrosterone sulfate), and testicular androgens (testosterone) (*234*). Testosterone, which is synthesized by the Leydig cells of the testis in most mammalian species, is the major circulating androgen in the male (*235*). Endogenous androgen is responsible for the development of sexual dimorphism in mammary development in rodents (*44*). During embryonal life, the target tissue for androgen is the mammary mesenchyme, which undergoes condensation under the influence of androgen secreted by the animal's own testes (*44*). Before the advent of antiestrogens, androgens were used for the treatment of advanced breast cancer. Androgens display both an estrogenic and an antiestrogenic effect, depending upon the nature of the receptor they activate (*236*). There is evidence that androgens behave as full estrogens when they bind to the estrogen receptor. At high concentrations, dehydrotestosterone induces estrogen-specific responses and stimulates the growth of DMBA-induced rat mammary tumors. Other androgens, like the 5-androstene-3β,17β-diol metabolite of the adrenal DHEA and 5α androstane-3β,17β-diol, which have a high affinity for the estrogen receptor, are estrogenic at near physiological concentrations and, through this mechanism, might stimulate the growth of hormone-dependent mammary cells (*236*). Androgens exert an antiestrogenic effect through the inhibition of the estrogen-induced increase in progesterone receptor (*236*). These various effects could explain the contradictory observations on the effect of androgens on mammary tumorigenesis reported in the literature. DMBA-induced rat mammary tumors, which regress after hypophysectomy and ovariectomy, also regress after treatment with the androgenic steroids testosterone, dihydrotestosterone, 2-methyldihydrotestosterone propionate, various enol derivatives of dihydrotestosterone, dromostanolone propionate, and 2-methyl-17β-hydroxy-5α-androstan-3-one (*25,27,237–241*). When moderate to high doses of androgenic steroids are given to rats with DMBA-induced tumors, an occasional mammary neoplasm is often enhanced, perhaps owing to partial conversion of the androgenic steroids to estrogenic agonists (*101,240*). Androgen inhibition of DMBA-induced carcinoma growth in rat mammae can be reversed by high doses of prolactin (*241*). Addition of testosterone to cell cultures of DMBA-induced mammary carcinomas inhibits DNA synthesis (*242*). Studies on the interactions of androgens with different receptors in estrogen target tissues have provided some information on the mechanisms of action of androgens in hormone-dependent tumors. The mechanism of the estrogenic activity of the androgens seems to be fairly well understood. It is known that androgens such as testosterone can be aromatized into estrogen, for example, in the hypothalamus. Androgens might also displace free active estrogens from their plasma binding protein, and they might act as full estrogens through their efficient interaction with the estrogen receptor. In contrast, the mechanism by which androgens are able to act as antiestrogens is not yet fully understood (*236*).

Thyroid Hormones

Thyroid hormones are synthesized by the thyroid gland under the regulation of the anterior pituitary hormone thyroid stimulating hormone (TSH). Thyroid hormones have major actions in almost all tissues; they control essential functions such as the regulation of energy metabolism and protein synthesis (*243*). Alterations in thyroid function, namely hypothyroidism, have been associated with a greater incidence of breast cancer (*244–247*). A statistically significant linear trend in the odds ratio for breast cancer has been found in women with subnormal levels of circulating free thyroxine (T_4) with long duration of ovulatory activity (*248*) and in women receiving thyroid medication for fertility problems (*249*); an excess in numbers of deaths from breast cancer has been reported in women with nontoxic nodular goiter (*250*). However, the number of studies is insufficient for drawing definitive conclusions on this relationship, and not all the studies support these conclusions (*246,247*).

In rodent models it has been found that thyroid hormones have a direct effect on growth and differentiation of the mammary gland (*243–247*). This effect is attributed in part to the induction of an increased sensitivity of the tissues to estradiol and prolactin through modulation of the number and the affinity characteristics of their respective receptors (*247*). Hypothyroidism in mice is associated with delayed lobuloalveolar development and a greater degree of postlactational involution of the mammary gland. Hyperthyroidism leads to increased development of the mammary tissue; however, it does not affect the postlactational phase of mammary gland regression (*244–247*). The fact that thyroid function also affects pituitary and ovarian function makes it difficult to determine the specific effects of thyroid hormone excess or insufficiency on mammary gland development and carcinogenesis. Studies performed in primiparous mice, made mildly hypothyroid by ingestion of thiouracil or mildly hyperthyroid by administration of thyroxine have revealed that the estrous cycles remain normal, and the levels of prolactin and the morphology and degree of mammary gland development in both groups of animals are similar to those of euthyroid animals. However, the incidence of spontaneous tumors is significantly lower in the hypothyroid animals at 1 year after removal of the pups (*246,247*). It is not clear whether thyroid hormones have a positive or a negative influence on mammary gland development and on carcinogen-induced mammary carcinomas in the rat (*247*). Conflicting results include the improved mammary gland development in castrated or estrogen-treated castrated rats after thyroidectomy. These effects were counteracted by T_4 administration. These observations suggest that the hypothyroid condition increases the sensitivity of the mammary glands to estrogens (*247*). With respect to mammary tumorigenesis, various studies have shown enhancement, inhibition, or no effect in rats made hyperthyroid by administration of thyroid hormones or hypothyroid by administration

of goitrogens. Despite the large number of studies (*101*), the direct or indirect influence of thyroid hormones on the development, growth, and progression of induced rat mammary carcinomas is still unresolved .

Current Knowledge in Human Breast Carcinogenesis

Human Breast Development

Two important concepts in breast development are that this organ is one of few that is not completely developed at birth and that it reaches its full differentiation only through the hormonal stimuli induced by pregnancy and lactation (*147*). The study of breast development reveals that the breast of postpubertal nulliparous women is composed of lobular structures reflecting different stages of development. Lobules type 1, also called terminal ductal lobular unit (TDLU), are the most undifferentiated ones; they are composed of clusters of 6–11 ductules per lobule. Lobules type 2 evolve from the previous ones and have a more complex morphology, being composed of a higher number of ductular structures per lobule. During pregnancy, lobules type 1 and type 2 progress to lobules type 3, which are characterized by having an average of 80 ductules or alveoli per lobule (Fig. 20). Lobules type 4, which are present only during the lactational period of the mammary gland, regress to type 3 after weaning (*147*). The structure most frequently found in the breast of nulliparous women of all ages is the lobule type 1, which comprises 50–60% of the total lobular component, followed in frequency by the lobules type 2 (30–35%); lobules type 3 are the least frequent (5–10%). In the breast of premenopausal parous women, on the other hand, lobules type 3 predominate, comprising 80–100% of the total lobular component (*149,150, 251*). These data are relevant to the fact that women with a history of early pregnancy have a 0.5 relative risk (RR) of developing breast cancer in comparison with nulliparous women (RR = 1.0). This effect is attributed to a greater degree of glandular differentiation induced by the reproductive process (*41,226,252,253*). We have shown that there are significant differences in the content and relative percentage of lobular structures present in the breast according to the parity status of a woman. In nulliparous women, lobules type 1 and type 2 are almost constantly present throughout their lifespan; the lobules type 2 decrease in number after menopause. In the parous woman's breast, the lobules type 3 are the most frequent structures present. Only after the fourth decade of life is there an increase in the number of lobules type 1 due to the regression of the more differentiated lobules type 3. At the end of the fifth decade of life, the breast of both nulliparous and parous women contains predominantly lobules type 1 (*251*). The undifferentiated structures, namely lobules type 1 and 2, which predominate in the breast of nulliparous women, also exhibit a high rate of cell proliferation, whereas the more differentiated lobules type 3 present in the breast of parous women exhibit a lower proliferative index (*147*).

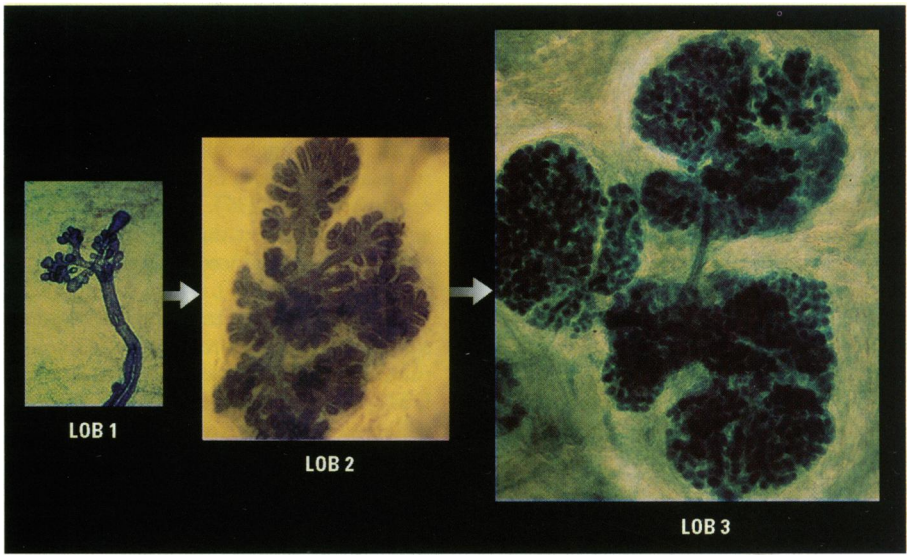

Figure 20. Whole mount preparations of sexually mature female breasts showing the three main lobular structures: the least differentiated lobules type 1 (LOB 1; ×99), which progress to lobules type 2 (LOB 2; ×118), and these progress to lobules type 3 (LOB 3; ×121). Toluidine blue.

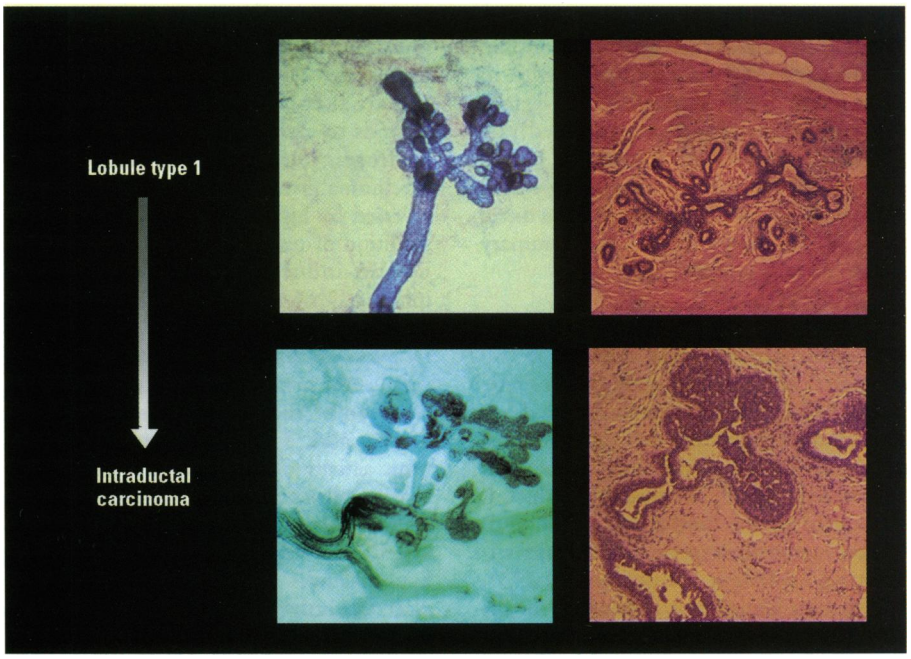

Figure 21. Lobules type 1, present in the breast of nulliparous women [whole mount (×105) and histological section (×118), upper panels], give origin to ductal carcinoma *in situ* [whole mount (×94) and histological section (×135), lower panels]. Whole mounts toluidine blue; histological sections H&E.

Pathogenesis of Human Breast Cancer

An important concept that emerged from our study of breast develoment is that the lobules type 1, or TDLU, have been identified as the site of origin of ductal carcinoma *in situ* (Fig. 21) (*23,254*). Supporting evidence to this observation is based on a comparative study of autopsies performed in women with and without breast cancer (*23*) in which we found that, in cancer-associated breasts, the number of hyper-

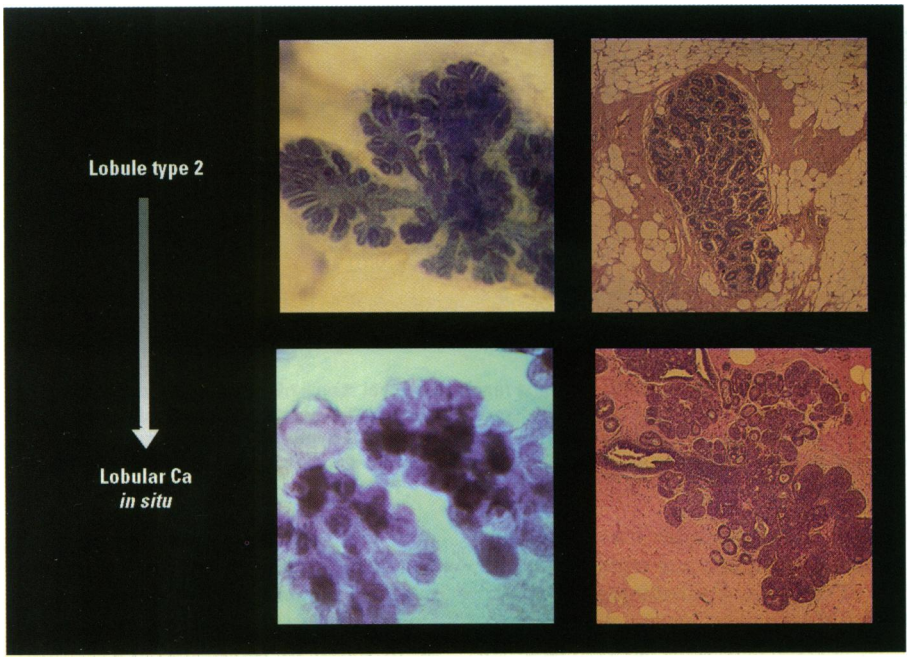

Figure 22. Lobules type 2 [whole mount (×105) and histological section (×105), upper panels] give origin to lobular carcinoma (Ca) *in situ* [whole mount (×135) and histological section (×94), lower panels]. Whole mounts, toluidine blue; histological sections, H&E.

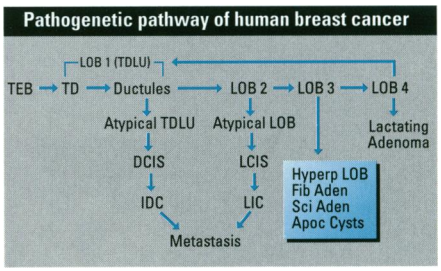

Figure 23. Pathogenetic pathway of human breast cancer. The terminal end bud (TEB) described in the virgin rat mammary gland is considered to be equivalent to the lobule type 1 (LOB 1) or terminal ductal lobular unit (TDLU) of the woman's breast. TD, terminal ducts; LOB 2, lobule type 2; LOB 3, lobule type 3; LOB 4, lobule type 4; DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma; LCIS, lobular carcinoma *in situ*; LIC, lobular invasive carcinoma; Hyperp LOB, hyperplastic lobule; Fib aden, fibroadenoma; Sci aden, sclerosing adenosis; Apoc cysts, apocrine cysts. From Russo et al. (*23*).

plastic terminal ducts, atypical lobules type 1, and ductal carcinoma *in situ* originated in lobules type 1 were significantly higher than in autopsies of women free of breast cancer (Fig. 21). An additional finding of these studies was that lobules type 2 give rise to the lobular carcinoma *in situ* (Fig. 22), whereas lobules type 3 and 4 originate more benign breast lesions (Fig. 23) (*23,149,150*). We concluded from these observations that each specific compartment of the breast gives origin to a specific lesion.

Transformation of Human Breast Epithelial Cells

The time of initiation of the carcinogenic process in women is not known. The observation that early full-term pregnancy is protective and that a higher incidence of mammary carcinomas occurred in women exposed to ionizing radiation at ages younger than 19 (*146*), but not after pregnancy and lactation (*22,60*) strongly suggests that, in the human female, the period between menarche and first full-term pregnancy might be critical for the initiation of breast carcinogenesis. Evidence documented in the previous section indicates that tumors originate in the terminal ductal structures most distal from the nipple (*22,60,254*). However, for answering the critical question of whether the lobules type 1 and type 2 are more susceptible than lobules type 3 to carcinogenesis, we have developed an *in vitro* system for manipulating the conditions of the breast epithelium that mimic the *in vivo* situation. For this purpose we have used mammoplasty specimens obtained from women that underwent voluntary surgery for breast reduction (*122,148*). Fresh tissues were digested with collagenase and hyaluronidase and the epithelial components of the gland were isolated as organoids. These structures were separated by micromanipulation and lobules type 1, 2, or 3 were identified, depending on the branching pattern or number of ductules per lobular unit, and placed in culture. The behavior *in vitro* varied depending upon the lobule type plated. Lobules type 1 and 2 attached to the dishes promptly and started growing in a logarithmic fashion, whereas the lobules type 3 had a long lag phase before they attached and started growing. The proliferative activity of these lobular structures, measured by uptake of ^{3}H-thymidine and expressed as DNA-labeling index, was higher in both lobules type 1 and 2 than in lobules type 3. In *in vitro* conditions, the number of doublings per unit of time was higher in lobules type 1 and 2 versus lobules type 3 (*255,256*). Primary cultures derived from organoids representing the lobules type 1, 2, and 3 were treated while in the log phase of growth with the chemical carcinogens MNU, DMBA, methyl-nitro-*N*-nitrosoguanidine (MNNG), or benzo(*a*)pyrene (BP) for 24 hrs. The cells were followed for several passages until the first evidence of transformation such as changes in cell morphology, loss of contact inhibition, and anchorage independent growth became evident. The carcinogens induced changes in cell shape by increasing the number of surface microvilli and by decreasing the cell–cell interaction. The typical domes that are characteristic of normal breast epithelial cells were lost when the cells were treated with the carcinogens, an effect attributed to the induction of alterations in contact inhibition and pattern of growth. When these cells were plated in agar methocel, they formed colonies (Fig. 24) (*148*). Of 52 human breast samples studied, the most important features found were the increased ability of the breast epithelial cells to survive in and form colonies in agar methocel and the expression of multinucleation. However, the response was only observed in epithelial cells derived from breast tissues containing lobules type 1 and 2. These phenomena were not observed in breast cells derived from lobules type 3 (*148,255*).

We concluded from these studies that primary cultures of human breast epithelial cells are affected by chemical carcinogenes, which induce transformation phenotypes whose expression depends upon the degree of gland development and *in vivo* cell proliferation of the donor's breast (*148,255*). The finding that lobules type 1 and 2 are more susceptible to express phenotypical changes of transformation *in vitro* supports our previous observations that lobules type 1 and type 2 are the site of origin of carcinomas, whereas lobules type 3, which are not associated with the development of malignant lesions, are not affected by carcinogens *in vitro*; this further emphasizes the concept that gland differentiation has a

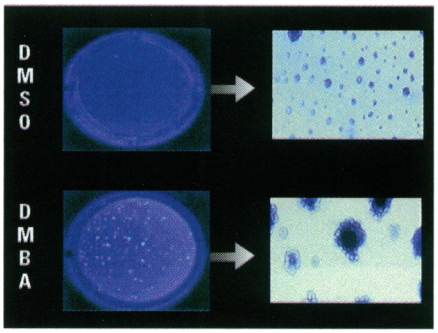

Figure 24. Primary cultures of human breast epithelial cells obtained from lobules type 1 form colonies in agar methocel after *in vitro* treatment with DMBA. Control cells treated with dimethyl sulfoxide (DMSO) do not form colonies. Colonies are stained with neutral red.

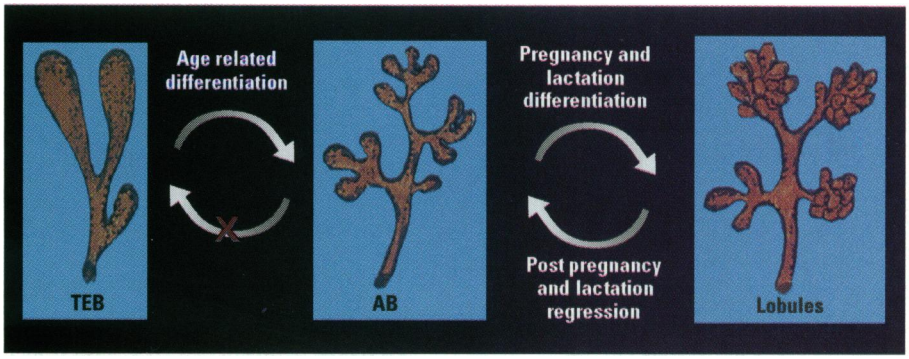

Figure 25. Terminal end buds (TEB), under the regular hormonal stimuli of the menstrual cycle, differentiate into alveolar buds (AB) in the nulliparous female. Pregnancy and lactation further the differentiation of AB to lobules. Arrows indicate unidirectional differentiation of TEB into AB and lobules. Postlactational involution does not restore the undifferentiated conditions of AB and TEB. From Russo and Russo (*41*).

protective effect *in vivo* and that this effect is also manifested in cells *in vitro*, rendering them resistant to carcinogen-induced neoplastic transformation.

Unified Concept of Mammary Carcinogenesis

Comparative studies of humans and rodents have allowed us to determine that mammary cancer originates in undifferentitated terminal structures of the mammary gland. The terminal ducts of the lobules type 1 of the human female breast, the site of origin of ductal carcinomas, have many points in common with the terminal end buds of the rat and mouse mammary gland, the site of origin of rodent mammary carcinomas. Cell replication in the lobules type 1 of the human breast has its highest peak during early adulthood at a time during which the breast is more susceptible to carcinogenesis. TEBs of the rat mammary gland also have their highest proliferative activity, binding of carcinogen to the DNA, and low repair capabilities when the animals are young and more susceptible to chemical carcinogens. Both in humans and in rodents, the mammary gland proliferative activity and susceptibility to carcinogenesis decrease with age. We have been able to demonstrate that the carcinogen acts on the TEB and that this structure is the one that evolves to intraductal proliferation, carcinoma *in situ*, and invasive carcinoma (Fig. 12). The undifferentiated lobule type 1 of the human breast has also the highest proliferative activity and *in vitro* binding of the carcinogen to the DNA and, more importantly, it expresses phenotypes of cell transformation after this treatment. These data indicate that in both rodents and humans the compartment in which the target or stem cell is found is the determinant factor in the initiation event. Several factors regulate the susceptibility of the stem cells to neoplastic transformation, namely, the topographic location of the mammary gland, age, and reproductive history of the host. Epidemiologic findings support this concept, because a higher incidence of breast carcinoma has been reported in nulliparous women and in women having an early menarche, factors that are indicative of a higher susceptibility to breast cancer, which is also coincident with the higher susceptibility to carcinogens observed in young virgin rats due to the presence of undifferentiated structures in the mammary tissue such as TEBs (Fig. 25). Thus, the protection afforded by early pregnancy and late menarche in humans or by pregnancy or treatment with pregnancy-related hormones such as hCG in rodents drives the mammary gland irreversibly towards the differentiation pathway (Fig. 25) instead of the neoplastic transformation pathway (Fig. 26). The relevance of our work lies in the validation of the rodent model system for understanding human breast cancer and the demonstration that both differentiation and cell proliferation, which are important in the initiation of carcinogenesis, are parameters that can be modulated for developing strategies for breast cancer prevention.

Experimental Rodent Mammary Tumor Models in Risk Assessment

Humans and all living species are exposed either acutely or chronically to numerous physical and chemical agents that can cause health hazards. The effects of those exposures can be manifested immediately, as acute toxic reactions, or can take years. In this latter case, the association of disease development to genotoxic agent exposure might become blurred, first, because of lack of awareness on the part of the patient or the treating physician that the exposure occurred, second, that the course of the disease might have been modified by endogenous and exogenous factors which might have acted synergystically or might have inhibited certain aspects of the process, and third, that even though exposure to a given agent might be recollected and acknowledged by both patient and physician, that agent might not have been recognized to be causative of disease or a genotoxic agent. The identification of specific physical and chemical agents as causative of human disease, namely cancer, and the demonstration in experimental systems that those agents can be mutagenic and/or carcinogenic have led to the development of methods for monitoring human exposure to given mutagens and carcinogens, and several biomarkers have been developed for this purpose (*257,258*). Epidemiologic studies have proven to be invaluable tools for identifying agents responsible of the causation of human disease, such as benzene in acute myelogenous leukemia (AML) (*259,260*), thus allowing their classification as known human carcinogens (EPA Class A, IARC Class I). The knowledge of the health effects of these known carcinogens has led the U.S. Occupational Safety and Health Administration (OSHA) to adopt specific requirements for risk assessment, biomonitoring and adoption of safety levels of exposure (*259*). However, epidemiological studies are limited because observations on the end effects of given physical or chemical agents may be performed several years after exposure and only relatively high risks will be detected. One additional problem is that epidemiologic data reveal historical exposures but do not provide information on the potential risk of new or untested chemicals.

The need to evaluate health risks associated with toxic chemical exposure has led to the development of risk assessment as an organized approach for 1) hazard identification for toxic chemicals and potential health effects that might occur in exposed popula-

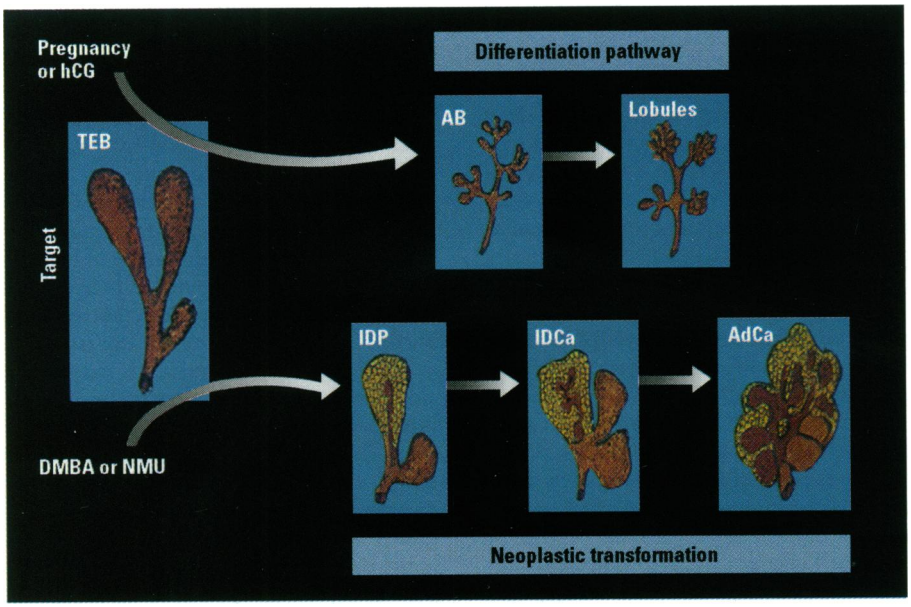

Figure 26. Terminal end buds (TEB) evolve to alveolar buds (AB) or lobules if pregnancy or human chorionic gonadotropin (hCG) stimulate them towards the differentiation pathway. If a carcinogen reaches the target (TEB) during the susceptibility period, it diverts this evolution to the neoplastic transformation pathway, developing instead into intraductal proliferation (IDP), intraductal carcinoma (IDCa), and invasive adenocarcinoma (AdCa). From Russo and Russo (*41*).

tions; 2) dose–response modeling; 3) exposure assessment; and 4) risk characterization. In 1976, the U.S. Environmental Protection Agency (EPA) adopted risk assessment and risk management as a two-step process for evaluating risk and policy development for reduction of exposure, respectively (*7*). The use of rodent animal models for hazard identification has proven to be useful for carcinogenicity testing. Rodent models are advantageous because they are informative in the absence of human data, they allow the assessment of risk, testing of dose effects, and the characterization of risk; however, the extrapolation of carcinogenic data obtained in rodents to human risk requires us to address basic concerns when evaluating unknown chemicals. First, it needs to be determined whether a tumorigenic response elicited in a rodent model by a specific treatment is a credible indicator for potential human risk. Proper statistical evaluation and mathematical analysis are required for determining the predictive value of a treatment-induced tumorigenic response (*261*). The assessment of the carcinogenic potential of an unknown agent requires the use of a model in which the multifactorial conditions that affect a human being during interaction with a given chemical are reproduced. This requirement should be better fulfilled by *in vivo* than by *in vitro* models. The ideal condition would be a model in which an animal is subjected to the same conditions as the human, for example, household pets. Cats that develop spontaneous mammary carcinomas which metastasize, like in humans (*50*), and dogs that develop mammary cancer in response to progestogens (*49*) have been proposed as adequate models. Monkeys, which are more similar to humans than any other species in menstrual cyclicity and endocrinologic conditions (*51*), should represent the optimal model; however, time, space, and economical restraints have hindered the wider use of these models in favor of the more economical rodent models. Rodent models of chemically induced mammary carcinogenesis have provided valuable information on the optimal host conditions, as well as on dose and route of administration to be utilized for eliciting maximal tumorigenic response (*12–20,25–29*). Knowledge gained through the study of these models has allowed researchers to determine that tumor incidence, number of tumors per animal, tumor type, and latency period of chemically induced mammary neoplasms are dose dependent, as well as dependent upon the age and reproductive history of the animal at the time of carcinogen exposure (*12–20,25–29,52*). Because known genotoxic agents are highly tumorigenic when given to young animals and the induced mammary neoplasms have a short latency period, the background induced incidence of spontaneous mammary tumors in rodents, which, although variable, develops late in life, becomes less relevant. These models have allowed researchers to test the influence of dose in the tumorigenic response. Carcinogen dose has been found to inversely affect tumor latency and directly affect the overall tumor incidence and number of tumors per animal; this effect has been observed exclusively for malignant but not for benign tumors (*32*). Thus, it can be concluded that treatment-related increases in the incidence and number of mammary carcinomas per animal are dose dependent, rather than a reflection of the maximum tolerated dose (MTD) used for routine toxicity testing.

Studies of normal mammary gland development and chemically induced rodent mammary carcinogenesis have provided useful information for clarifying how the interplay of ovarian, pituitary, and placental hormones, while influencing the development and physiology of the mammary gland, modulate its response to given chemical carcinogens (*12–22,25–29, 38–42,64–66*). Rodent experimental models have been useful for testing the hormone dependence of mammary tumors. Although the role played by the most important endogenous hormones in the development, progression, and regression of rodent mammary neoplasia has been fairly well characterized (*43,101,244–247*), the fact that they are essential elements in normal body functions implies a no-adverse effect level threshold for the normal effects and the interplay of feedback mechanisms (*262*). Hormones may not be carcinogenic in themselves, but they may allow the neoplastic transformants initiated by proximal carcinogens to establish and grow by modifying the host or target tissue (*115*) or through a variety of mechanisms, such as the activation of viruses, induction of receptors for carcinogens, or the initiation of DNA synthesis following carcinogenic insult. A point of concern in evaluating the cancer risk posed by any given substance is the role played by the route of exposure. The literature available on the carcinogenic potential of direct- and indirect-acting genotoxic agents indicates that route of exposure is fairly specific for each given type of agent. Indirect-acting liposoluble polycyclic hydrocarbons are traditionally administered intragastrically (*27,18–22*), whereas the direct-acting MNU has to be rapidly injected intravenously (*12–14,101*). The fact that topical applications of DMBA in the mammary gland and intraperitoneal injections of MNU elicit a tumorigenic response indicates that alternative routes may also be indicative of the carcinogenic potential of a given substance. However, the large variability in tumorigenic response reported by different laboratories using identical carcinogens, doses, and routes and

even intralaboratory variations makes it difficult to generalize on the appropriateness of route selection for carcinogenic testing and risk assessment. Despite these concerns, careful comparisons of the pathogenesis and histopathology of mammary cancer in humans and rodents have provided a reliable basis for extrapolating carcinogenic data from experimental models to the human condition (*23,24*).

Conclusions and Future Directions

This work describes and compares experimental *in vivo* models presently used for assessing the mammary carcinogenic potential of given chemical and physical agents and addresses the need for identifying those models that provide information on the mechanisms involved in cancer initiation and progression in the human population. The main problems identified in this study are the complexity and multistep nature of the carcinogenic process, the lack of understanding of the mechanism(s) involved in the initiation and progression of the disease, and the lack of identification of a specific etiologic agent or agents of human breast cancer. Long-term studies in rodent models have been traditionally used, mainly due to the susceptibility of the rodent mammary gland to develop both spontaneous and chemically induced neoplasms. The usefulness of chemically induced mammary tumors lies in their hormone dependence; the high frequency of adenocarcinomas histologically similar to human breast cancers; the possibility they offer for analyzing the initiation, promotion, and progression steps of carcinogenesis; and the baseline information they provide in risk assessment. Classical studies of these models have allowed researchers to identify the optimal host conditions, as well as the dose and route of administration to be used for eliciting maximal tumorigenic response. Chemically induced mammary tumors develop by a multistep process, which begins as a biochemical lesion caused by the interaction of the carcinogen with cellular DNA. In this interaction, the DNA is damaged and, if the damage is not repaired efficiently, the result is a mutation, chromosomal translocation, inactivation of regulatory genes, or more subtle changes that are not yet well identified. Neoplastic development requires that the lesion becomes fixed, aided by cell proliferation, and progresses to a third stage of autonomous growth, which results in cancer when the lesion acquires the capacity to invade and metastasize. Several carcinogens such as MCA, DMBA, and MNU have been extensively studied in mice and rats. DMBA and MNU have been the most frequently used; the majority of the mammary tumors induced in rats by either one of these agents are hormone-dependent adenocarcinomas. The observation that maximal tumor incidence is elicited when the carcinogens are administered to young virgin female rats has led to important discoveries on the role of mammary gland development and differentiation in cancer initiation. Hormonally induced differentiation and the interplay of ovarian, pituitary, and placental hormones have been identified as powerful modulators of the tumorigenic response elicited by genotoxic chemicals.

Humans and all living species are exposed either acutely or chronically to numerous physical and chemical agents that can cause health hazards. The human population is exposed to a large number of environmental chemicals such as polycyclic aromatic hydrocarbons, nitrosoureas, and aromatic amines that are known to be carcinogenic in *in vivo* experimental animal models and are known to induce mutagenesis and neoplastic transformation of mammary cells in *in vitro* models. The potential of chemicals to induce cancer in humans has been recognized for over two centuries. The identification of specific physical and chemical agents such as radiation, coal tar, and benzene as causes of human cancers and leukemias has led to the classification of some agents as known human carcinogens (EPA Class A, IARC Class I). The demonstration that these agents can be mutagenic or carcinogenic in experimental systems has constituted the basis for developing biomarkers for monitoring human exposure. The knowledge of the health effects of these known carcinogens has led OSHA to adopt specific requirements for risk assessment, biomonitoring, and adoption of safety levels of exposure (*7*). Confirmation of these effects through epidemiological studies, however, is limited because observations on the end effects of given physical or chemical agents might be performed several years after exposure and only relatively high risks will be detected. Because epidemiological data reveal historical exposures but do not provide information on the potential risk of new or untested chemicals, experimental animal models should provide the necessary information for accurately predicting the human carcinogenic potential of a given substance. The utilization of rodent animal models for hazard identification has proven to be useful for carcinogenicity testing. Rodent models are advantageous because they are informative in the absence of human data, they allow the assessment of risk, the testing of dose effects and the characterization of risk. However, the extrapolation of carcinogenic data obtained in rodents to human risk requires basic concerns be addressed when evaluating unknown chemicals. First, it should be determined whether a tumorigenic response elicited in a rodent model by a specific treatment is a credible indicator for potential human risk. Proper statistical evaluation and mathematical analysis are required for determining the predictive value of a treatment-induced tumorigenic response. The assessment of the carcinogenic potential of an unknown agent requires the use of a model in which the multifactorial conditions that affect a human being during interaction with a given chemical are occurring. The relevance of experimental animal models should be validated by careful comparisons with the human condition. The greater the similarities between the two systems, the greater the predictive value and the validity of extrapolations from rodents to humans (*263–266*).

References

1. Dorland's illustrated medical dictionary. 25th ed. Philadelphia, PA:W.B. Saunders, 1974.
2. Rubin E, Farber JL, eds. Pathology. Philadelphia:J.B. Lippincott, 1994.
3. American Cancer Society. Selected cancers. Cancer facts and figures, 1994. Atlanta, GA:American Cancer Society, 1994;9–17.
4. Wingo PA, Tong T, Bolden S. Cancer Statistics, 1995. Ca Cancer J Clin 45:8–30 (1995).
5. Kelsey JL, Horn-Ross PL. Breast cancer: magnitude of the problem and descriptive epidemiology. Epidemiol Rev 15:7–16 (1993).
6. Steele GD Jr, Osteen RT, Winchester DP, Murphy GP, Menck HR. Clinical highlights from the National Cancer Data Base: 1994. Ca Cancer J Clin 44:71–78 (1994).
7. Anderson EL. Cancer risk assessments: an overview. In: Oncogene and transgenics correlates of cancer risk assessments. (Zervos C, ed). New York:Plenum Press, 1992;1–20.
8. Finch C. Reproductive senescence in rodents: factors in the decline of fertility and loss of regular estrous cycles. In: The aging reproductive system (Schneyder E, ed). New York:Raven Press, 1978; 193–212.
9. Welsch CW. Rodent models to examine *in vivo* hormonal regulation of mammary gland tumorigenesis. In: Cellular and molecular biology of mammary cancer (Medina D, Kidwell W, Heppner G, Anderson E, eds). New York:Plenum Press, 1987;163–179.
10. Callahan R. Retrovirus and proto-oncogene involvement in the etiology of breast neoplasia. In: The mammary gland (Neville MC, Daniel CW, eds). New York:Plenum Press, 1987; 323–351.
11. Teich N, Wyke J, Mak T, Bernstein A, Hardy W. Pathogenesis of retrovirus-induced disease. In: RNA tumor viruses, (Weiss R, Teich N, Varmus H, Coffin J, eds). Cold Spring Harbor, NY:Cold Spring Harbor Laboratory, 1982;785–998.
12. Grubbs CJ, Farnell DR, Hill DL, McDonough

KC. Chemoprevention of *N*-nitroso-*N*-methylurea-induced mammary cancers by pretreatment with 17β-estradiol and progesterone. J Natl Cancer Inst 4:927–932 (1985).
13. Grubbs CJ, Hill DL, McDonough KC, Peckham JC. *N*-nitroso-*N*-methylurea-induced mammary carcinogenesis: effect of pregnancy in preneoplastic cells. J Natl Cancer Inst 71:625–628 (1983).
14. Grubbs CJ, Juliana MM, Hill DL, Whitaker LM. Suppression by pregnancy of chemically induced preneoplastic cells of the rat mammary gland. Anticancer Res 6:2395–2401 (1986).
15. Russo J, Saby J, Isenberg W, Russo IH. Pathogenesis of mammary carcinoma induced in rats by 7,12-dimethylbenz(*a*)anthracene. J Natl Cancer Inst 59:435–445 (1977).
16. Russo IH, Russo J. Developmental stage of the rat mammary gland as determinant of its susceptibility to 7,12-dimethylbenz(*a*)anthracene. J Natl Cancer Inst 61:1439–1449 (1978).
17. Russo J, Russo IH. DNA labeling index and structure of the rat mammary gland as determinant of its susceptibility to carcinogenesis. J Natl Cancer Inst 61:1451–1459 (1978).
18. Russo J, Wilgus G, Russo IH. Susceptibility of the mammary gland to carcinogenesis. I. Differentiation of the mammary gland as determinant of tumor incidence and type of lesion. Am J Pathol 96:721–736 (1979).
19. Russo J, Russo IH. Influence of differentiation and cell kinetics on the susceptibility of the rat mammary gland to carcinogenesis. Cancer Res 40:2677–2687 (1980).
20. Russo J, Russo IH. Susceptibility of the mammary gland to carcinogenesis. II. Pregnancy interruption as a risk factor in tumor incidence. Am J Pathol 100:497–512 (1980).
21. Russo J, Tay LK, Russo IH. Model systems to study breast carcinogenesis. In: Laboratory medicine. Advances in Pathology, vol 2 (Levy E, ed). Oxford:Pergamon Press, 1981;27–30.
22. Russo J, Tay LK, Russo IH. Differentiation of the mammary gland and susceptibility to carcinogenesis. Breast Cancer Res Treat 2:5–37 (1982).
23. Russo J, Gusterson BA, Rogers AE, Russo IH, Wellings SR, Van Zwieten MJ. Comparative study of human and rat mammary tumorigenesis. Lab Invest 62:1–32 (1990).
24. Russo J, Russo IH, van Zwieten MJ, Rogers AE, Gusterson B. Classification of neoplastic and non-neoplastic lesions of the rat mammary gland. In: Integument and mammary glands of laboratory animals (Jones TC, Mohr U, Hunt RD, eds). Berlin:Springer-Verlag, 1989; 275–304.
25. Huggins C. Two principles in endocrine therapy of cancers; hormone deprival and hormone interference. Cancer Res 25:1163–1167 (1965).
26. Huggins C, Grand LC, Brillantes FP. Critical significance of breast structure in the induction of mammary cancer in the rat. Proc Natl Acad Sci USA 45:1294–1300 (1959).
27. Huggins C, Lorraine C, Grand LC, Brillantes FP. Mammary cancer induced by a single feeding of polynuclear hydrocarbons and its suppression. Nature 189:204–207 (1961).
28. Huggins C, Moon RC, Moni S. Extinction of experimental mammary cancer. I. Estradiol-17β and progesterone. Proc Natl Acad Sci USA 48:379–386 (1962).
29. Huggins C, Yang NC. Induction and extinction of mammary cancer. Science 137:257–262 (1962).
30. McCormick DL, Moon RC. Retinoid-tamoxifen interaction in mammary cancer chemoprevention. Carcinogenesis 7:193–196 (1986).
31. McCormick DL. Anticarcinogenic activity of quinacrine in the rat mammary gland. Carcinogenesis 9:175–178 (1988).
32. McCormick DL, Adamowski CB, Fiks A, Moon RC. Lifetime dose–response relationships for mammary tumor reduction by a single administration of *N*-methyl-*N*-nitrosourea. Cancer Res 41:1690–1694 (1981).
33. McCormick DL, Burns FJ, Albert RE. Inhibition of benzo(*a*)pyrene-induced mammary carcinogenesis by retinyl acetate. J Natl Cancer Inst 66:559–564 (1981).
34. McCormick DL, Mehta RG, Thompson CA, Dinger N, Caldwell JA, Moon RC. Enhanced inhibition of mammary carcinogenesis by combined treatment with *n*-(4-hydroxyphenyl) retinamide and ovariectomy. Cancer Res 42:508–512 (1982).
35. Moon RC, McCormick DL, Mehta RG. Inhibition of carcinogenesis by retinoids. Cancer Res(suppl) 43:2469s–2475s (1983).
36. Medina D, Shepherd F. Selenium-mediated inhibition of 7,12-dimethylbenz(*a*)anthracene induced mouse mammary tumorigenesis. Carcinogenesis 2:451–455 (1981).
37. Tornqvist M, Ehrenberg L. On cancer risk estimation of urban air pollution. Environ Health Perspect 102(suppl 4):173–182 (1994).
38. Russo J, Wilgus G, Tait L, Russo IH. Influence of age and parity on the susceptibility of rat mammary gland epithelial cells in primary cultures to 7,12-dimethylbenz(*a*)anthracene. In Vitro 17:877–884 (1981).
39. Russo IH, Medado J, Russo J. Endocrine influences on mammary structure and development. In: Integument and mammary gland of laboratory animals (Jones TC, Mohr U, Hunt RD, eds). Berlin:Springer Verlag, 1989;252–266.
40. Russo IH, Tewari M, Russo J. Morphology and development of rat mammary gland. In: Integument and mammary gland of laboratory animals (Jones TC, Mohr U, Hunt RD, eds). Berlin:Springer-Verlag, 1989;233–252.
41. Russo J, Russo IH. Toward a physiological approach to breast cancer prevention. Cancer Epidemiol Biomarkers Prev 3:353–364 (1994).
42. Daniel CW, Silberstein GB. Postnatal development of the rodent mammary gland. In: The mammary gland (Neville MC, Daniel CW, eds). New York:Plenum Press, 1987;3–36.
43. Meyer F, Brisson J, Morrison AS, Brown JB. Endogenous sex hormones, prolactin, and mammographic features of breast tissue in premenopausal women. J Natl Cancer Inst 77:617–620 (1986).
44. Sakakura T. Mammary embryogenesis. In: The mammary gland (Neville MC, Daniel CW, eds). New York:Plenum Press, 1987;37–66.
45. MacKenzie WF, Garner FM. Comparison of neoplasms in six sources of rats. J Natl Cancer Inst 50:1234–1257 (1973).
46. Solleveld HA, Haseman JK, McConnell EE. Natural history of body weight gain, survival, and neoplasia in the F344 rat. J Natl Cancer Inst 72:929–940 (1984).
47. Zwieten MJ. Normal anatomy and pathology of the rat mammary gland. In: The rat as animal model in breast cancer research. Boston:Martinus Nijhoff, 1984;53–134.
48. Pike MC, Spicer DV, Dahmoush L, Press MF. Estrogens, progestogens, normal breast cell proliferation, and breast cancer. Epidemiol Rev 15:17–35 (1993).
49. Selman PJ, Mol JA, Rutterman GR, Garderen EV, Rijnberk A. Progestin-induced growth hormone excess in the dog originates in the mammary gland. Endocrinology 134:287–292 (1994).
50. Stolwijk JAM, Minke JMHM, Rutteman GR, Hoekstra J, Prop FJA, Misdorp W. Feline mammary carcinomas as a model for human breast cancer. II. Comparison of *in vivo* and *in vitro* adriamycin sensitivity. Anticancer Res 9:1045–1048 (1989).
51. Broerse JJ, van Bekkum DW, Zoetelief J, Zurcher C. Relative biological effectiveness for neutron carcinogenesis in monkeys and rats. Radiat Res S128–S135 (1991).
52. Dao TL. The role of ovarian hormones initiating the induction of mammary cancer in rats by polynuclear hydrocarbons. Cancer Res 22:973–980 (1962).
53. Hummel KP, Richardson FL, Feketo E. Anatomy. In: Biology of the laboratory mouse (Green EL, ed). New York:McGraw Hill Book Company, 1966;247–307.
54. Russo J. Basis of cellular autonomy in susceptibility to carcinogenesis. Toxicol Pathol 11:149–163 (1983).
55. Russo IH, Russo J. From pathogenesis to hormone prevention of mammary carcinogenesis. Cancer Surv 5:649–670 (1986).
56. Russo J, Isenberg WM, Ireland M, Russo IH. Ultrastructural changes in the mammary epithelial cell population during neoplastic development induced by chemical carcinogen. Proc Electron Microsc Soc Am 34:250–251 (1976).
57. Russo IH, Ireland W, Russo J. Ultrastructural description of three different epithelial cell types in rat mammary gland. Proc Electron Microsc Soc Am 34:146–147 (1976).
58. Russo J, Wells P. Ultrastructural localization of adenosine triphosphatase activity in resting mammary gland. J Histochem Cytochem 25:135–148 (1977).
59. Williams JM, Daniel CW. Mammary ductal elongation: differentiation of myoepithelium and basal lamina during branching morphogenesis. Dev Biol 97:274–290 (1983).
60. Russo J, Russo IH. Biological and molecular bases of mammary carcinogenesis. Lab Invest 57:112–137 (1987).
61. Ghadially FN. Diagnostic electron microscopy of tumors. London:Butterworth and Company, 1985.
62. Gusterson BA, Warburton MJ, Monaghan P. Histologic/immunocytochemical markers, mammary gland, rat. In: Integument and mammary glands (Jones TC, Mohr U, Hunt RD, eds). Berlin:Springer Verlag, 1989; 266–274.
63. Warburton MJ, Mitchell D, Ormerod EJ, Rudland P. Distribution of myoepithelial cells and basement membrane proteins in the resting, pregnant, lactating, and involuting rat mammary gland. J Histochem Cytochem 30:667–676 (1982).
64. Raynaud A. Observationns sur le developpement normal des ebauches de la glande mammaire des foetus males et femellas de souris. Ann Endocrinol 8:349–359 (1947).

65. Raynaud A. Action des hormones sixuelles sur le developpement embryonnaire de la glande mammaire de la souris. Comptb Rend Soc Biol 225:150–152 (1947).
66. Dickson RB, Bano M, Johnson MD, Shi YE, Martinez-Lacaci I, Amundadottir LT, Ziff B, Kurebayashi J. Steroid regulation of growth factors and protooncogenes in the normal and malignant mammary gland. In: Protooncogenes and growth factors in steroid hormone induced growth and differentiation (Khan SA, Stancel GM, eds). Boca Raton, FL:CRC Press, 1994;143–174.
67. Vonderhaar BK. Regulation of development of the normal mammary gland by hormones and growth factors. In: Breast cancer: cellular and molecular biology (Lippman ME, Dickson RB, eds). Boston:Kluwer, 1988;251–294.
68. Peluso JJ. Morphologic and physiologic features of the ovary. In: Pathobiology of the aging rat (Mohr U, Dungworth DL, Capen CC, eds). Washington, DC:ILSI Press, 1992;337–349.
69. Russo IH, Russo J. Role of hCG and inhibin in breast cancer. Int J Oncol 4:297–306 (1994).
70. Woodruff TK, Mayo KE. Regulation of inhibin synthesis in the rat ovary. Annu Rev Physiol 52:807–827 (1990).
71. Yung SY. Inhibins, activins and follistatins: gonadal proteins modulating the secretion of follicle-stimulating hormone. Endocrine Rev 9:267–293 (1988).
72. Chatterton RT. Progesterone and mammary gland development. In: The sex steroids (McKerns KW, ed). New York:Appleton-Century-Crofts, 1971.
73. Dembinski TC, Shiu RPC. Growth factors in mammary gland development and function. In: The mammary gland (Neville MC, Daniel CW, eds). New York:Plenum Press, 1987;355–382.
74. Tonelli QJ, Sorof S. Expression of a phenotype of normal differentiation in cultured mammary glands is promoted by epidermal growth factor and blocked by cyclic adenine nucleotide and prostaglandins. Differentiation 20:253–259 (1981).
75. Sirbasku DA. Estrogen induction of growth factors specific for hormone-responsive mammary, pituitary, and kidney tumor cells. Proc Natl Acad Sci USA 75:3786–3790 (1978).
76. Haslam SZ. Role of sex steroid hormones in normal mammary gland function. In: The mammary gland (Neville MC, Daniel CW, eds). New York:Plenum Press, 1987;499–535.
77. Kuhn NJ. Lactogenesis: the search for trigger mechanisms in different species. In: Comparative aspects of lactation (Peaker M, ed). New York:Academic Press, 1977; 165–192.
78. Nandi S. Endocrine control of mammary gland development and function in the C3H/He Crgl mouse. J Natl Cancer Inst 21:1039–1063 (1958).
79. Mizuno H, Natio M. The role of prolactin in the mammary alveolus formation. Endocrinl JPN 2:163–169 (1956).
80. Russo IH, Russo J. Hormone prevention of mammary carcinogenesis: a new approach in anticancer research. Anticancer Res 8: 1247–1264 (1988).
81. King MM, McCoy P, Russo IH. Dietary fat may influence DMBA-initiated mammary gland carcinogenesis by modification of mammary gland development. In: Current topics in nutrition and disease (Roe, Daphne A ed). New York:Alan R. Liss, 1983;61–90.
82. Arts CJ, Glovers CA, van Den Berg H, Thijssen JH. Effect of wheat bran and energy restriction on onset of puberty, cell proliferation and development of mammary tissue in female rats. Acta Endocrinol 126:451–459 (1992).
83. Wiehle RD, Wittliff JL. Alterations in sex-steroid hormone receptors during mammary gland differentiation in the rat. Comp Biochem Physiol 76BL:409–417 (1983).
84. Hwang JJ, Lee AB, Fields PA, Haab LM, Mojonnier LE, Sherwood OD. Monoclonal antibodies specific for rat relaxin. V. Passive immunization with monoclonal antibodies throughout the second half of pregnancy disrupts development of the mammary apparatus and, hence, lactational performance in rats. Endocrinology 129:3034–3042 (1991).
85. Alvarado MV, Russo J, Russo IH. Immunolocalization of inhibin in the mammary gland of rats treated with hCG. J Histochem Cytochem 41:29–34 (1993).
86. Alvarado ME, Russo J, Russo IH. Comparative study of inhibin expression in the mammary gland of pregnant and human chorionic gonadotropin (hCG) treated rats. Proc Am Assoc Cancer Res 34:1444a. (1993).
87. Jahn GA, Edery M, Belair L, Kelly PA, Dijiane J. Prolactin receptor gene expression in rat mammary gland and liver during pregnancy and lactation. Endocrinology 128:2976–2984 (1991).
88. Helminen HJ, Ericsson JLE, Orremius S. Studies on mammary gland involution. IV. Histochemical and biochemical observations on alterations in lysosomes and lysosomal enzymes. J Ultrastruct Res 25:240–252 (1968).
89. Ciocca DR, Parente A, Russo J. Endocrinologic milieu and susceptibility of the rat mammary gland to carcinogenesis. Am J Pathol 109:47–56 (1982).
90. Wise PM. The role of the hypothalamus in aging of the female reproductive system. J Steroid Biochem 27:713–719 (1987).
91. Demarest KT, Moore KE, Riegle GD. Adenohypophyseal dopamine content and prolactin secretion in the aged male and female rat. Endocrinology 116:1316–1323 (1985).
92. Peng MT, Huang HH. Aging of hypothalamic-pituitary-ovarian function in the rat. Fertil Steril 23:535–542 (1972).
93. Felicio LS, Nelson JF, Gosden RG, Finch RE. Restoration of ovulatory cycles by young ovarian graft in aging mice: potentiation by long term ovariectomy decreases with age. Proc Soc Exp Biol Med 169:348–354 (1982).
94. Peng MT, Peng YM. Changes in the uptake of irridiated estradiol in the hypothalamus and adenohypophyisis of old female rats. Fertil Steril 24:534–539 (1974).
95. Haji M, Kato K, Nawata H, Ibayashi H. Age-related changes in concentrations of cytosol receptors for sex steroid hormones in the hypothalamus and pituitary gland of rats. Brain Res 204:373–386 (1981).
96. Kanungo MS, Patnaik SK, Koul O. Decrease in 17β-estradiol receptor in brain of aging rats. Nature 253:366–367 (1975).
97. Jrang MJ, Peng MT. Cytoplasmic and nuclear binding of estradiol in the brain pituitary of old female rats. Gerontology 27:51–57 (1981).
98. Baulieu E-E, Kelly PA, eds. Hormones: from molecules to disease. New York:Chapman and Hall, 1990.
99. Brooks CM, Koizumi K. The hypothalamus and control of integrative processes. In: Medical physiology (Mountcastle VB, ed). St. Louis, MO:C.V. Mosby, 1980;1:923–947.
100. Cowie AT, Folley SJ. The role of the adrenal cortex in mammary development and its relation to the mammogenic action of the anterior pituitary. Endocrinology 40:274–285 (1947).
101. Welsch CW. Host factors affecting the growth of carcinogen-induced rat mammary carcinomas: a review and tribute to Charles Brenton Huggins. Cancer Res 45:3415–3443 (1985).
102. Stefaneanu L, Kovacs K. Changes in structure and function of the pituitary. In: Pathobiology of the aging rat, vol 2 (Mohr U, Dungworth DL, Capen CC, eds). Washington, DC:ILSI Press, 1994;174–191.
103. Vessey MP. Exogenous hormones in the etiology of cancer in women. J Roy Soc Med 77:542–549 (1984).
104. Kelly PA, Posner BI, Tsushima T, Friessen HG. Studies of insulin, growth hormone and prolactin binding: ontogenesis, effects of sex and pregnancy. Endocrinology 95:532–539 (1974).
105. Meites J. Relation of prolactin and estrogen to mammary tumorigenesis in the rat. J Natl Cancer Inst 48:1217–1224 (1972).
106. Horvath E, Kovacs K. The adenohypophysis. In: Functional endocrine pathology, vol 1 (Kovac K, Asa SL, eds). Boston:Blackwell Scientific Publications 1991;245–281.
107. Brawer JR, Beaudet A, Desjardins GC, Schipper HM. Minireview: pathologic effect of estradiol on the hypothalamus. Biol Reprod 49:647–652 (1993).
108. Finch C, Felicio LS, Mobbs CV, Nelson JF. Ovarian and steroidal influences on neuroendocrine aging processes in female rodents. Endocr Rev 5:467–497 (1984).
109. Czernobilsky B, Czernobilsky H. The female reproductive tract. In: functional endocrine pathology, vol 2 (Kovac K, Asa SL, eds). Boston:Blackwell Scientific Publications, 1991;608–651.
110. Kanungo MS, Patnaik SK, Koul O. Decrease in 17β-estradiol receptor in brain of aging rats. Nature 53:366–367 (1975).
111. Sherman BM, Korenman SG. Inadequate corpus luteum function: patophysiological interpretation of human breast cancer epidemiology. Cancer 33:1306–1312 (1974).
112. Wetzel LT, Luempert LC 3rd, Breckenridge CB, Tisdel MO, Stevens JT, Thakur AK, Extrom PJ, Eldridge JC. Chronic effects of atrazine on estrus and mammary tumor formation in female Sprague-Dawley and Fischer 344. J Toxicol Environ Health 43:169–182 (1994).
113. Goodman HM. Basic medical endocrinology. New York:Raven Press, 1994;271–295.
114. McConnell RF. Comparative aspects of contraceptive steroids: effects observed in rats. Toxicol Pathol 21:3294–3295 (1989).
115. Heywood R. The toxicological assessment of the safety of hormonal contraception. In: Safety requirements for contraceptive steroids (Michel F, ed). New York:Cambridge University Press, 1989;159–165.
116. Rao GN, Piegorsch WW, Haseman JK. Influence of body weight on the incidence of spontaneous tumors in rats and mice of long-term studies. Am J Clin Nutr 45:252–260 (1987).

117. Bittner JJ. The milk influence on breast tumors in mice. Science 95:462–463 (1942).
118. Lang PL. Spontaneous neoplastic lesions and selected non-neoplastic lesions in the Crl:CD BR rat. Wilmington, MA:Charles River Laboratories, 1992.
119. Haseman JF, Huff J, Boorman GA. Use of historical control data in carcinogenicity studies in rodents. Toxicol Pathol 12:126–135 (1984).
120. Ross MH, Bras G. Lasting influence of early caloric restriction on prevalence of neoplasms in the rat. J Natl Cancer Inst 47:1095–1113 (1971).
121. Tarone RE, Chu KC, Ward JM. Variability in the rates of some common naturally occurring tumors in Fischer 344 rats and (C57BL/6N × C3/HeN) F_1 ($B6C3F_1$) mice. J Natl Cancer Inst 66:1175–1181 (1981).
122. Russo J, Calaf G, Sohi N, Tahin Q, Zhang PL, Alvarado ME, Estrada S, Russo IH. Critical steps in breast carcinogenesis. Ann NY Acad Sci 698:1–20 (1993).
123. Hancock SL, Tucker MA, Hoppe RT. Breast cancer after treatment of Hodgkin's disease. Ann J Natl Cancer Inst 85:25–31 (1993).
124. BEIR: Committee on the Biological Effects of Ionizing Radiations, National Research Council. Somatic effects: Cancer. In: The effects on populations of exposure to low levels of ionizing radiation. Washington, DC:National Academy of Science, 1980;135–158.
125. Land CE, Boice JD, Shore RE, Norman JE, Tokunaga M. Breast cancer risk from low-dose exposures to ionizing radiation: results of parallel analysis of three exposed populations of women. J Natl Cancer Inst 65:353–376 (1980).
126. Finerty JC, Binhammer RT, Schneider M, Cunningham AWB. Neoplasms in rats exposed to single-dose total-body X-radiation. J Natl Cancer Inst 14:149–154 (1953).
127. Bond VP, Cronkite EP, Lippincott SW, Shellabarger CJ. Studies on radiation-induced mammary gland neoplasia in the rat. III. Relation of the neoplastic response to dose of total-body radiation. Radiat Res 12:276–284 (1960).
128. Bond VP, Shellabarger CJ, Cronkite EP, Fliedner TM. Studies on radiation-induced mammary gland neoplasia in the rat. V. Induction by localized irradiation. Radiat Res 13:318–325 (1960).
129. Broerse JJ, Hennen LA, Solleveld HA. Actuarial analysis of the hazard for mammary carcinogenesis in different rat strains after X- and neutron irradiation. Leuk Res 10:749–754 (1986).
130. van Bekkum DW, Broerse JJ, Hennen LA, Solleveld HA. The gene transfer–misrepair hypothesis of radiation carcinogenesis tested for induction of mammary tumors in rats. Leuk Res 10:761–765 (1986).
131. Shellabarger CJ. Pituitary and steroid hormones in radiation-induced mammary tumors. In: Hormones and breast cancer (Pike MC, Siteri PK, Welsch CW, eds). Cold Spring Harbor NY:Cold Spring Harbor Laboratory, 1981;339–351.
132. Shellabarger CJ, Bond BP, Aponte GE, Cronkite EP. Results of fractionation and protraction of total-body radiation on rat mammary neoplasia. Cancer Res 26:509–513 (1966).
133. Zwieten JF. Effects of irradiation and hormone administration on mammary tumor incidence. In: The rat as animal model in breast cancer research. Boston;Martinus Nijhoff, 1984;29–52.
134. Solleveld HA, van Zwieten MJ, Broerse JJ, Hollander CF. Effects of X-irradiation, ovariohysterectomy and estradiol-17β on incidence, benign/malignant ratio and multiplicity of rat mammary neoplasms—a preliminary report. Leuk Res 10:755–763 (1986).
135. Broerse JJ, Hennen LA, Klapwijk WM, Solleveld HA. Mammary carcinogenesis in different rat strains after irradiation and hormone administration. Int J Radiat Biol 51:1091–1100 (1987).
136. Segaloff A, Maxfield WS. The synergism between radiation and estrogen in the production of mammary cancer in the rat. Cancer Res 31:166–168 (1971).
137. Stone JP, Holtzman S, Shellabarger CJ. Synergistic interactions of various doses of diethylstilbestrol and X-irradiation on mammary neoplasia in female ACI rats. Cancer Res 40:3966–3972 (1980).
138. Shellabarger CJ, Stone JP, Holtzman S. Rat differences in mammary tumor induction with estrogen and neutron radiation. J Natl Cancer Inst 61:1505–1508 (1978).
139. Holtzman S, Stone JP, Shellabarger CJ. Influence of diethylstilbestrol treatment on prolactin cells of female ACI and Sprague-Dawley rats. Cancer Res 39:779–784 (1979).
140. Yokoro K, Sumi C, Ito A, Hamada K, Kanda K, Kobsayashi T. Mammary carcinogenic effect of low-dose fission radiation in Wistar/Forth rats and its dependency on prolactin. J Natl Cancer Inst 64:1459–1466 (1980).
141. Blankenstein MA, Broerse JJ, van Zwieten MJ, van der Molen HJ. Prolactin concentration in plasma and susceptibility to mammary tumors in female rats from different strains treated chronically with estradiol-17β. Breast Cancer Res Treat 4:137–141 (1984).
142. Holtzman S, Stone JP, Shellabarger CJ. Radiation-induced mammary carcinogenesis in virgin, pregnant, lactating and post-lactating rats. Cancer Res 42:50–53 (1982).
143. Miki Y, Swensen J, Shattuck-Elden D, Futreal PA, Harshman K, Tavtigian S, Liu Q, Cochran C, Bennett LM, Ding W. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science 266:66–71 (1994).
144. MacMahon B, Cole P, Lin TM,Lowe CR, Mirra AP, Ravnihar B, Salbur EJ, Valaoras VG, Yuasa S. Age at first birth and breast cancer risk. Bull World Health Organ 43:209–221 (1970).
145. Russo J, Russo IH. Is differentiation the answer in breast cancer prevention? Int Res Commun Med Sci 10:935–945 (1982).
146. McGregor DH, Land CE, Choi K, Tokuoka S, Liu PI, Wakabayashi I, Beebe GW. Breast cancer incidence among atomic bomb survivors, Hiroshima and Nagasaki 1950–1989. J Natl Cancer Inst 59:799–811 (1977).
147. Russo J, Russo IH. Role of differentiation on transformation of human breast epithelial cells. In: Cellular molecular biology of experimental mammary cancer (Medina D, Kidwell W, Heppner G, Anderson E, eds). New York: Plenum Press, 1987;399–417.
148. Russo J, Calaf G, Russo IH. A critical approach to the malignant transformation of human breast epithelial cells. Crit Rev Oncog 4:403–417 (1993).
149. Russo J, Russo IH. Developmental pattern of the human breast and susceptibility to carcinogenesis. Eur J Cancer Prev 2:85–100 (1993).
150. Russo J, Romero AL, Russo IH. Architectural pattern of the normal and cancerous breast under the influence of parity. Cancer Epidemiol Biomarkers Prev 3:219–224 (1994).
151. Dao TL, Chan PC. Effect of duration of high fat intake on enhancement of mammary carcinogenesis in rats. J Natl Cancer Inst 71:201–208 (1983).
152. Ito N. *In vivo* carcinogeneisi of 4-nitroquinoline 1-oxide and related compounds. In: Carcinogenesis, vol. 6: the nitroquinolines (Sugimura T, ed). New York:Raven Press, 1981;117–153.
153. Shirai T, Fysh JM, Lee M-S, Vaught JB, King CM. Relationship of metabolic activation on *N*-hydroxy-*N*-acylarylamines to biological response in the liver and mammary gland of the female CD rat. Cancer Res 41:4346 (1981).
154. Young S, Hallowes RC. Tumours of the mammary gland. In: Pathology of tumors in laboratory animals, vol 1 (Turusov VS, ed). IARC scientific publication no. 5. Lyon:International Agency for Research on Cancer,1973;31–73.
155. Gullino PM, Pettigrew HM, Grantham FH. *N*-nitrosomethylurea as mammary gland carcinogen in rats. J Natl Cancer Inst 54:401–414 (1975).
156. Thompson HJ, Meeker LD. Induction of rat mammary gland carcinoma by the subcutaneous injection of 1 methyl-1-nitrosourea. Cancer Res 43:1628–1633 (1985).
157. Rogers AE, Lee SY. Chemically-induced mammary gland tumors in rats: modulation by dietary fat. In: Dietary fat and cancer (Ip C, Birt DF, Rogers AE, Mettlin C, eds). New York:Alan R. Liss, 1986;255–282.
158. Clinton SK, Imrey PB, Alster JM, Simon J, Truex CR, Visek WJ. The combined effects of dietary protein and fat on 7,12-dimethylbenz(*a*)anthracene-induced breast cancer in rats. J Nutr 114:1213–1218 (1984).
159. Welsch CW, DeHoog JV, O'Connor DH. Influence of caffeine consumption on carcinomatous and normal mammary gland development in mice. Cancer Res 48:2078–2082 (1988).
160. McCormick DL, Madigan MJ, Moon RC. Modulation of rat mammary carcinogenesis by indomethacin. Cancer Res 45:1803–1809 (1985).
161. Thompson HJ, Ronan A. Effect of D,L-α-difluoromethylornithine and endocrine manipulation on the induction of mammary carcinogenesis by 1-methyl-1-nitrosourea. Carcinogenesis 57:2003–2009 (1987).
162. Rose DP, Pruitt B, Stauber P, Erturk E, Bryan GT. Influence of dosage schedule on the biological characteristics of *N*-nitroso-*N*-methylurea-induced rat mammary tumors. Cancer Res 40:235–239 (1980).
163. Tay LK, Russo J. 7,12-Dimethylbenz(*a*)-anthracene (DMBA) induced DNA binding and repair synthesis in susceptible and nonsusceptible mammary epithelial cells in culture. J Natl Cancer Inst 67:155–161 (1981).
164. Tay LK, Russo J. Formation and removal of 7,12-dimethylbenz(*a*)anthracene nucleic acid adducts in rat mammary epithelial cells with

different susceptibility to carcinogenesis. Carcinogenesis 2:1327–1333 (1981).
165. Tay LK, Russo J. Effect of human chorionic gonadotropin on 7,12-dimethylbenz(*a*)anthracene-induced DNA binding and repair synthesis by rat mammary epithelial cells. Chem Biol Interact 55:13–21 (1985).
166. Chan PC, Dao TL. Effects of dietary fat on age-dependent sensitivity to mammary carcinogenesis. Cancer Lett 18:245–253 (1983).
167. Russo J, Tay LK, Ciocca D, Russo IH. Molecular and cellular basis of the mammary gland susceptibility to carcinogenesis. Environ Health Perspect 49:185–199 (1983).
168. Russo J, Tait L, Russo IH. Susceptibility of the mammary gland to carcinogenesis III. The cell of origin of mammary carcinoma. Am J Pathol 113:50–66 (1983).
169. Russo J, Russo IH. Boundaries in mammary carcinogenesis. In: Boundaries between promotion and progression during carcinogenesis. (Sudilovsky O, ed). New York:Plenum Press, 1991;43–59.
170. Hashimoto K, Tarnowski WM, Lever WF. Reifung und degranulierum der Matzellen in der merch. Lichen Haut Hautarzt 18:318–324 (1967).
171. Folkman J. How is blood vessel growth regulated in normal and neoplastic tissue? Cancer Res 46:467–473 (1986).
172. Dvorak HF, Dvorak AM. Basophils, mast cells and cellular immunity in animals and man. Human Pathol 3:454–456 (1972).
173. Moller L, Schuetzle D, Autrup H. Future research needs associated with the assessment of potential human health risks from exposure to toxic ambient air pollutants. Environ Health Perspect 102(suppl 4):193–210 (1994).
174. Isaacs JT. Genetic control of resistance to chemically induced mammary adenocarcinogenesis in the rat. Cancer Res 46:3958–3963 (1986).
175. Isaacs JT. Inheritance of a genetic factor from the Copenhagen rat and the suppression of chemically induced mammary adenocarcinogenesis. Cancer Res 48:2204–2213 (1988).
176. Rothschild TC, Boylan ES, Calhoon RE, Vonderhaar BK.Transplacental effects of diethylstilbestrol on mammary development and tumorigenesis in female ACI rats. Cancer Res 47:4508–4516 (1987).
177. Stone JP, Holtzman S, Shellabarger CJ. Neoplastic responses and correlated plasma prolactin levels in diethylstilbestrol-treated AC1 and Sprague-Dawley rats. Cancer Res 39:773–778 (1979).
178. Aylsworth CF, Cullum ME, Zile, MH, Welsch CW. Influence of dietary retinyl acetate on normal rat mammary gland development and on the enhancement of 7,12-dimethylbenzanthracene induced rat mammary tumorigenesis by high levels of dietary fat. J Natl Cancer Inst 76:339–345 (1986).
179. Rogers AE, Conner B, Boulanger C, Lee S. Mammary tumorigenesis in rats fed diets high in lard. Lipids 21:275–284 (1986).
180. Klurfeld DM, Weber MM, Kirtchevsky D. Inhibition of chemically induced mammary and colon tumor promotion by caloric restriction in rats fed increased dietary fat. Cancer Res 47:2759–2765 (1987).
181. Kritchevsky D, Weber MM, Klurfeld DM. Dietary fat versus caloric content initiation and promotion of 7,12-dimethylbenz(*a*)-anthracene-induced mammary tumorigenesis in rats. Cancer Res 44:3174–3177 (1984).
182. Beth M, Berger MR, Aksoy M, Schmahl D. Comparison between the effects of dietary fat level and of calorie intake on methylnitrosurea-induced mammary carcinogenesis in female SD rats. Int J Cancer 39:737–744 (1987).
183. Cohen LA, Thompson DO, Maeura Y, Choi K, Blank ME, Rose DP. Dietary fat and mammary cancer. Promoting effects of different dietary fat on *N*-nitrosomethylurea-induced rat mammary tumorigenesis. J Natl Cancer Inst 77:33–42 (1986).
184. Rogers AE, Conner BH, Boulanger C, Lee SY, Carr FA, Dumouchel WH. Enhancement of 7,12-dimethylbenz(*a*)anthracene mammary carcinogenesis by a high lard diet. In: Dietary fiber (Vahouny GV, Kritchevsky D, eds). Plenum Publishing, 1985;449–460.
185. Sylvester PW, Russell M, Ip MM, Ip C. Comparative effects on different animal and vegetable fats fed before and during carcinogen administration on mammary tumorigenesis, sexual maturation, and endocrine function in rats. Cancer Res 46:757–762 (1986).
186. Sylvester PW, Ip C, Ip M. Effects of high dietary fat on the growth and development of ovarian-independent carcinogen-induced mammary tumors in rats. Cancer Res 46:763–769 (1986).
187. Wetsel WC, Rogers AE, Newberne PM. Dietary fat and DMBA mammary carcinogenesis in rats. Cancer Detect Prev 4:535–547 (1981).
188. Lee SY, Walsh CT, Ng SF, Rogers AE. Toxicokinetics of 7,12-dimethylbenz(*a*)anthracene (DMBA) in rats fed high lard or control diets. J Nutr Growth Cancer 3:167–173 (1986).
189. Wetsel WC, Rutledge A, Rogers AE. Absence of an effect of dietary corn oil content on plasma prolactin, progesterone and 17β-estradiol in female Sprague-Dawley rats. Cancer Res 44:1420–1425 (1983).
190. Lee SY, Ng SF, Busby WF, Rogers AE. Mammary gland DNA synthesis in rats fed hog lard or control diets. J Nutr Growth Cancer 4:167–174 (1987).
191. Kort WJ, Weijma IM, Bijma AM, van Schalkwijk WP, Vergroesen AJ, Westbroek DL. Omega-3 fatty acids inhibiting the growth of a transplantable rat mammary adenocarcinoma. J Natl Cancer Inst 79:593–599 (1987).
192. Welsch CW, DeHoog JV, O'Connor DH. Influence of caffeine and/or coffee consumption on the initiation and promotion phases of 7,12-dimethylbenz(*a*)anthracene-induced rat mammary gland tumorigenesis. Cancer Res 48:2068–2073 (1988).
193. Welsch CW, DeHoog JV. Influence of caffeine consumption on 7,12-dimethylbenz(*a*)-anthracene-induced mammary gland tumorigenesis in female rats fed a chemically defined diet containing standard and high levels of unsaturated fat. Cancer Res 48:2074–2077 (1988).
194. Gapstur SM, Potter SD, Sellers TA, Folsom AR. Increased risk of breast cancer with alcohol consumption in postmenopausal women. Am J Epidemiol 136:1221–1231 (1992).
195. Rogers AE, Longnecker MP. Dietary and nutritional influences on cancer: a review on epidemiologic and experimental data. Lab Invest 59:729–759 (1988).
196. Meites J. Farm animals: hormonal inductions of lactation and galactopoiesis. In: Milk: the mammary gland and its secretions (Kon SK, Cowie AT, eds). New York:Academic Press, 1961;321–367.
197. Leclercq G, Heuson JC. Physiological and pharmacological effects of estrogens in breast cancer. Biochem Biophys Acta 560:427–455 (1979).
198. Geschickter CF. The mechanism of tumor formation. In: Diseases of the breast. Philadelphia, PA:J.B. Lippincott, 1945;774–799.
199. Meites J. Relation of prolactin and estrogen to mammary tumorigenesis in the rat. J Natl Cancer Inst 48:1217–1223 (1972).
200. Stouffer RL. Corpus luteum function and dysfunction. Clin Obstet Gynecol 33:668–689 (1990).
201. Jabara AG. Effects of progesterone on 9,12-dimethyl-1,2-benzanthracene-induced mammary tumors in Sprague-Dawley rats. Br J Cancer 21:418–429 (1967).
202. Jabara AG, Harcourt AG. The effects of progesterone and ovariectomy on mammary tumors induced by 7,12-dimethylbenzathracene in Sprague-Dawley rats. Pathology 2:115–123 (1970).
203. Jabara AG, Harcourt AG Effect of progesterone, ovariectomy and adrenalectomy on mammary tumors induced by 7,12-dimethylbenzathracene in Sprague-Dawley rats. Pathology 3:209–214 (1971).
204. Jabara AG, Toyne PH, Harcourt AG. Effects of time and duration of progesterone administration on mammary tumors induced by 7,12-dimethylbenzathracene in Sprague-Dawley rats. Br J Cancer 27:63–71 (1973).
205. Shay H, Harri C, Gruenstein M. Influence of sex hormones on the incidence and form of tumors produced in male or female rats by gastric instillation of methylcholanthrene. J Natl Cancer Inst 13:307–332 (1952).
206. Matzuk MM, Finegold MJ, Su JGJ, Husueh AJW, Bradley A. Alpha inhibin is a tumor suppressor gene with gonadal specificity in mice. Nature 360:313–319 (1992).
207. Manni A, Trujillo JE, Pearson OH. Predominant role of prolactin in stimulating the growth of 7,12-dimethylbenz(*a*)-anthracene-induced rat mammary tumors. Cancer Res 37:1216–1219 (1977).
208. Nagasawa H. Prolactin: its role in the development of mammary tumors. Med Hypotheses 5:1117–1121 (1979).
209. Nagasawa H, Morii S. Prophylaxis of spontaneous mammary tumorigenesis by temporal inhibition of prolactin secretion in rats at young ages. Cancer Res 41:1935–1937 (1981).
210. Welsch CW. Prolactin and the development and progression of early neoplastic mammary gland lesions. Cancer Res 38:4054–4058 (1978).
211. Welsch CW, Brown CK, Goodrich-Smith M, Van J, Denenberg B, Anderson T, Brooks CL. Inhibition of mammary tumorigenesis in carcinogen-treated Lewis rats by suppression of prolactin secretion. J Natl Cancer Inst 63:1211–1214 (1979).
212. Swanson SM, Guzman RC, Christov K, Miyamoto S, Nandi S. Pituitary-isografted mice are highly susceptible to MNU-induced mammary carcinogenesis irrespective of the level of alveolar differentiation. Carcinogenesis 15:1341–1346 (1994).
213. Welsch CW, Nagasawa H. Prolactin and murine mammary tumorigenesis: a review. Cancer Res 37:951–963 (1977).
214. Formelli F, Zaccheo T, DiSalle E, Ornati G, DiMarco A. Correlation between inhibitor

effects on prolactin secretion and antitumor activity of new ergoline compounds on DMBA-induced tumors in rats. Eur J Cancer Clin Oncol 19:1545–1551 (1983).
215. Kamberi IA, Mical RS, Porter JC. Effect of anterior pituitary perfusion and intraventricular injection of catacholamines and indoleamines on LH release. Endocrinology 87:1–12 (1970).
216. Soto AM, Sonnenschein C. Estrogen receptor levels in estrogen sensitive cells in culture. J Steroid Biochem 11:1185–1190 (1979).
217. Brawer JR, Sonnenschein C. Cytopathological effects of estradiol on the accurate nucleus of the female rat. A possible mechanism for pituitary tumorigenesis. Am J Anat 144:57–88 (1975).
218. Berkvens JM, van Nesselrovy JHJ, Kroes R. Spontaneous tumors in the pituitary gland of old Wistar rats. A morphological and immunocytochemical study. J Pathol 130:179–191(1980).
219. MacLeod RM. Regulation of prolactin secretion. In: Frontiers in neuroendocrinology, (Martini L, Ganong WF, eds). New York: Raven Press, 1976;164–194.
220. Yen SSC. Clinical endocrinology of reproduction. In: Hormones: from molecules to disease (Baulieu E-E, Kelly PA, eds). New York: Chapman and Hall, 1990;445–482.
221. Russo IH, Koszalka MS, Russo J. Protective effect of chorionic gonadotropin on DMBA-induced mammary carcinogenesis. Br J Cancer 62:243–247 (1990).
222. Nisula BC, Taliadouros GS, Carayon P. Primary and secondary biologic activities intrinsic to the human chorionic gonadotropin molecule. In: Chorionic gonadotropin (Segal SJ, ed). New York:Plenum Press, 1980;17–35.
223. Wide L, Hobson B, Wide M. Chorionic gonadotropin in rodents. In: Chorionic gonadotropin (Segal SJ, ed). New York:Plenum Press, 1980;37–51.
224. Russo IH, Koszalka M, Russo J. Human chorionic gonadotropin and mammary cancer prevention. J Natl Cancer Inst 82:1286–1289 (1990).
225. Russo IH, Koszalka M, Russo J. Effect of human chorionic gonadotropin on mammary gland differentiation and carcinogenesis. Carcinogenesis 11:1849–1855 (1990).
226. Russo IH, Russo J. Physiological basis of breast cancer prevention. Eur J Cancer Prev 2:101–111 (1993).
227. Faulkner A, Peaker M. Regulation of mammary glucose metabolism in lactation. In: The mammary gland: development, regulation and function. (Neville MC, Daniel CW, eds). New York:Plenum Press, 1987;535–562.
228. Topper JY, Nicholas KR, Sankaran L, Kulski J. Insulin as a developmental hormone. In: Hormones and cancer (Gurpide E, Calandra R, Levy C, Soto RJ, eds). New York:Alan R. Liss, 1984;63–77.
229. Heuson JC, Legros N. Study of the growth-promoting effect of insulin in relation to carbohydrate metabolism in organ culture of rat mammary carcinoma. Eur J Cancer 4:1–7 (1968).
230. Heuson JC, Legros N. Effect of insulin and of alloxan diabetes on growth of the rat mammary carcinoma *in vivo*. Eur J Cancer 6:349–351 (1970).
231. Heuson JC, Legros N. Influence of insulin deprivation on growth of the 7,12-dimethylbenzanthracene-induced mammary carcinoma in rats subjected to alloxan diabetes and food restriction. Cancer Res 32:226–232 (1972).
232. Heuson NC, Legro N, Heimann R. Influence of insulin administration on growth of the 7,12-dimethylbenzathracene-induced mammary carcinoma in intact, oophorectomized, and hypophysectomized rats. Cancer Res 32:233–238 (1972).
233. Hilf R, Hissin PJ, Shafie SM. Regulatory interrelationships for insulin and estrogen action in mammary tumors. Cancer Res 38:4076–4085 (1978).
234. Milgrom E. Steroid Hormones. In: Hormones: from molecules to disease (Baulieu E-E, Kelly PA, eds). Paris:Hermann Publishers in arts and sciences (published simulteneously in Britain; London:Chapman and Hall) 1990;387–437.
235. Rommerts FFG. Testosterone: an overview of biosynthesis, transport, metabolism and action. In: Testosterone—action, deficiency, substitution (Nieschlag E, Behre HM, eds). Berlin: Springer-Verlag, 1990;1–22.
236. Rochefort H. Biochemical basis of breast cancer treatment by androgens and progestins. In: Hormones and cancer (Gurpide E, Calandra R, Levy C, Soto RJ, eds). New York:Alan R. Liss, 1984;79–95.
237. Ercoli A, Briziarelli G. Effect of enrol-ether derivatives of dihydrotestosterone on 3-methylcholanthrene-induced rat mammary carcinoma. J Natl Cancer Inst 27:1173–1187 (1961).
238. Griswold DP, Green CH. Observations on the hormone sensitivity of 7,12-dimethylbenzanthracene-induced mammary tumors in the Sprague-Dawley rat. Cancer Res 30:819–826 (1970).
239. Harada T, Rooks WH, Dorfman RI. An inhibitory effect of steroids on 20-methylcholanthrene and 7,12-dimethylbenzanthracene-induced rat mammary tumors. Oncologia 19:3–10 (1965).
240. Heise E, Gorlich M. Growth and therapy of mammary tumors induced by 7,12-dimethylbenzaanthracene in rats. Br J Cancer 20:539–545 (1966).
241. Quadri SK, Kledzik GS, Meites J. Counteraction by prolactin androgen-induced inhibition of mammary tumor growth in rats. J Natl Cancer Inst 52:875–878 (1974).
242. Shafie SM. Estrogen and the growth of breast cancer: new evidence suggests indirect action. Science 209:701–702 (1980).
243. Lissitzky S. Thyroid hormones. In: Hormones: from molecules to disease (Baulieu E-E, Kelly, PA, eds). Paris:Hermann Publishers in arts and sciences (published simulteneously in Britain; London:Chapman and Hall) 1990;343–386.
244. Vonderhaar BK. Hormones and growth factors in mammary gland development. In: Control of cell growth and proliferation (Venezial CM, ed), New York:Van Nostrand and Reinhold, 1984;11–33.
245. Vonderhaar BK. Prolactin: transport, function, and receptors in mammary gland development and differentiation. In: The mammary gland: development, regulation and function (Neville MC, Daniel CW, eds). New York:Plenum Press, 1987;383–438.
246. Vonderhaar BK, Greco AE. Effect of thyroid status on development of spontaneous mammary tumors in primiparous C3H mice. Cancer Res 42:4553–4561 (1982).
247. Vonderhaar BK. Effect of thyroid hormones on mammary tumor induction and growth. In: Hormonal regulation of experimental mammary tumors (Leung BS, ed). Elmsford, NY:Pergamon Press, 1982;138–154.
248. Morabia A, Szklo M, Stewart W, Schuman L, Thomas DB, Zacur HA. Thyroid hormones and duration of ovulatory activity in the etiology of breast cancer. Cancer Epidemiol Biomarkers Prev 1:389–393 (1992).
249. Brinton LA, Hoffman. DA, Hoover R, Fraumeni JF Jr. Relationship of thyroid disease and use of thyroid supplements to breast cancer risk. J Chronic Dis 37:877–893 (1984).
250. Goldman MB, Monson RR, Maloof F. Cancer mortality in women with thyroid disease. Cancer Res 50:2283–2289 (1990).
251. Russo J, Rivera R, Russo IH. Influence of age and parity on the development of the human breast. Breast Cancer Res Treat 23:211–218 (1992).
252. De Waard F, Trichopoulos D. A unifying concept of the aetiology of breast cancer. Int J Cancer 41:666–669 (1988).
253. John EM, Kelsey JL. Radiation and other environmental exposures and breast cancer. Epidemiol Rev 15:157–162 (1993).
254. Wellings SR, Jensen HM, Marcum RG. An atlas of subgross pathology of 16 human breasts with special reference to possible precancerous lesions. J Natl Cancer Inst 55:231–275 (1975).
255. Russo J, Reina D, Frederick J, Russo IH. Expression of phenotypical changes by human breast epithelial cells treated with carcinogens *in vitro*. Cancer Res 48:2837–2857 (1988).
256. Russo J, Mills MJ, Moussalli MJ, Russo IH. Influence of breast development and growth properties *in vitro*. In Vitro Cell Dev Biol 25:643–649 (1989).
257. Anwar WA. Monitoring of human populations at risk by different cytogenetic end points. Environ Health Perspect 102(suppl 4):131–134 (1994).
258. Connolly RB, Andersen ME. Biologically-based pharmacodynamic models: tools for toxicological research and risk assessment. Ann Rev Pharmacol Toxicol 31:503–523 (1991).
259. Crump KS. Risk assessment for benzene induced leukemia. In: Oncogene and transgenics correlates of cancer risk assessment (Zervos C, ed). New York:Plenum Press, 1992;241–262.
260. Moltholt B. A threshold for benzene leukemogenesis. In: Oncogene and transgenics correlates of cancer risk assessment (Zervos C, ed). New York:Plenum Press, 1992;305–312.
261. Kokoska SM.The analysis of cancer chemoprevention experiments. Biometrics 43:525–534 (1987).
262. Paynter OE, Burin GJ, Jaeger RB, Gregorio CA. Goitrogens and thyroid follicular cell neoplasia. Evidence for a threshold process. Regul Toxicol Pharmacol 8:102–119 (1988).
263. Ashby J, Tennant RW. Prediction of rodent carcinogenicity for 44 chemicals:results. Mutagenesis 9:7–15 (1994).
264. Barrett JC. Mechanisms of multistep carcinogenesis and carcinogen risk assessment. Environ Health Perspect 100:9–20 (1993).
265. Lang L. Mouse or molecule? Mechanism-based toxicology in cancer risk assessment. Environ Health Perspect 103:334–336 (1995).
266. Sutter TR. Molecular and cellular approaches to extrapolation for risk assessment. Environ Health Perspect 103:386–389 (1995).